STUDY ON THE INTERSPECIFIC HYBRIDIZATION OF PLEUROTUS BY PROTOPLAST FUSION

BY

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ABSTRACT

The purpose of this project was to study the interspecific hybridization between Pleurotus sajor-caju and P. florida by means of fusion of protoplasts . The distinctive macroscopic and microscopic characteristics between these two species were investigated. The fruit body (sporophore) morphology of P. sajor-caju was quite different from that of P. florida, including fruit body colour and growth habit. From the study on mating tests and cytological observation, it could be concluded that hyphal fusion did not occur between these two species. Auxotrophic mutants, obtained by UV light mutagenesis of basidiospores, were used as selective markers for the recognition of interspecific heterokaryons derived by protoplast fusion .

Spherical, osmotically sensitive protoplasts were isolated from mycelia through the action of a combination of Novozym 234 and Cellulase CP in a suitable osmotic solution. Good protoplast yields, about 10 per ml, were obtained from young mycelia at an early- exponential growth phase by the use of 0.6 M $MgSO_4$ in a 0.01 M phosphate buffer at pH 5.8. Two patterns of morphological development of the protoplasts were observed in osmotic liquid medium: one was a chain of yeast-like

structure and the other, a normal hypha. Among the stabilizers tested, mannitol appeared to be more suitable for Pleurotus protoplast reversion. There appeared to be no significant differences in the reversion frequency of protoplasts grown on the complete medium and on the minimal medium. However, the reversion frequency was generally low and only 4-5% was observed.

Polyethylene glycol (PEG) at a concentration of 30%, was used, as a fusogen , in the fusion of protoplasts from auxotrophic mutants of the two Pleurotus species to produce interspecific heterokaryons. The fusion frequency ranged from 2% to 12%. The cells of the isolated interspecific heterokaryons generally had two nuclei which could have been contributed by both parental fusion partners through protoplast fusion to fulfill the nutrient complementation. From sexual backcrosses of the interspecific heterokaryons to the parental testers, it was found that nonrandom or preferential selection of the two nuclear components in the heterokaryons occurred during the establishment of derived dikaryons. From the analysis of the isoenzyme banding patterns of alcohol dehydrogenase and esterase from the interspecific heterokaryons, it might be concluded that the genomes of both species did exist in the same cell and their differential interaction gave the various expression in the genotype. However, the interspecific heterokaryons

failed to form fruit bodies, although some did form pin-head primordia but these could not differentiate any further into fruit bodies.

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ABBREVIATIONS AND SYMBOLS

ADH	alcohol dehydrogenase
Bis	NN'-methylene bis-acrylamide
°C	degree celsius
cm	centimeter
EMS	ethylmethane sulphonate
erg	unit of energy (= 10^{-7} joule)
fwt	fresh weight
g	gramme
xg	centrifugal force
hr	hour
l	litre
nm	monometer
mA	milliampere
mg	milligramme
ml	millilitre
mM	millimolarity
min	minute
M	molarity
MW	molecular weight
NTG	N-methyl-N-nitrosoguanidine
NT	not tested
%	percent
rpm	rotation per minute

sec	second
TEMED	N,N,N',N'-tetramethylethylenediamine
Tris	2-amino-2-hydroxymethyl-1,3-propanediol
UV	ultraviolet light
w	watt

CHAPTER 1

GENERAL INTRODUCTION

During the last twenty years, workers from various fields of plant biology have recognized the great potentials which isolated protoplasts could offer when employed as a tool in experimental systems and in breeding programmes. The protoplast is the main part of a plant cell bounded by an outer cell wall. Isolated spherical protoplasts can be released after the removal of the cell wall in an osmotically stabilized solution.

The most frequently quoted potential use of protoplast fusion technology is the production of interspecific hybrids which would not have been possible to obtain by normal sexual means. Thus, somatic hybridization by means of protoplast fusion could provide useful genetic materials for research work, particularly in plant breeding. In fact, this technique has already been applied successfully to bacterial and fungal species leading to the isolation of recombinant progeny (Peberdy, 1980), and significant progress has also been achieved in some higher plants (Cove, 1979). Novel plants could be isolated from interspecific hybrid cell lines, obtained by means of protoplast fusion, involving species which were sexually

incompatible. These have already been achieved between some plant species such as Daucus carota + Agopodium podagraria (Dudits et al., 1979), Datura innoxia + Atropa belladonna (Krumbiegel and Achieder, 1979) and Lycopersicum esculentum + Solanum tuberosum (Shepard et al., 1983). Therefore, protoplast fusion has proved to be a valuable tool even for intra- and intergeneric-hybridization, at least among the plant genera studied.

The fundamental requirements for protoplast fusion are the consistently high yield of viable protoplasts, a good reversion frequency of protoplasts, an efficient protoplast fusion method, and suitable selective markers for the recovery of hybrids. Many workers have favoured the use of lytic enzymes for protoplast isolation, which is most efficient and reliable. This technique has now been established in many fungal species (Villanueva and Garcia-Acha, 1971; Peberdy, 1979). The success of this technique depends mainly on three critical factors: the lytic enzyme, the osmotic stabilizer and the physiological status of the organism (Peberdy, 1976). However, the establishment of suitable conditions for protoplast isolation from any new organism has to be a matter of trial and error, because of the complex interactions which exist among above mentioned factors. Fewer problems exist in the reversion of fungal protoplasts. The culture of fungal protoplasts on nutritive medium supple-

mented with a suitable osmotic stabilizer generally leads to a rapid regeneration of the cell wall and the reversion to normal mycelia.

The fusion of protoplasts is the critical step in the formation of a somatic hybrid and a number of methods have been developed for accomplishing this. Among them, the use of polyethylene glycol (PEG) as a fusogen is the most popular both in green plants and in fungi.

It is desirable to be able to recognize the fusion products as early as possible after the regeneration of fused protoplasts. Complementation of the auxotrophic mutant strains has been widely used in the selection of both intra- and interspecific hybrids in fungi. In most cases, auxotrophic mutants usually were recessive and could be easily detected in haploid organisms. Auxotrophs are more readily available in fungi because much of their life cycle is haploid phase. Mutation can occur spontaneously, but usually at an extremely low frequency. Almost all mutants used in experimental work have to be obtained by artificial induction. The most generally used mutagenic treatment is UV-irradiation, although various kinds of chemical mutagens (e.g., ethylmethane sulphonate (EMS), 5-bromuracil, etc.) have also often been used.

This study was divided into four major parts:

(1) The biological nature of Pleurotus sajor-caju and P.

florida. The main purpose was to investigate the morphological differences between these two species and their sexual compatibility.

(2) The induction and characterization of mutants. UV-irradiation was employed in the induction of auxotrophic mutants. The nutrient requirement, mating type, fructification ability and other biochemical expression of these isolated auxotrophic mutants were studied in detail. The identified and confirmed auxotrophs of the two Pleurotus species were used as the parental strains for protoplast fusion.

(3) Protoplast isolation and regeneration. Monokaryotic mycelia were used for the establishment of suitable conditions for Pleurotus protoplast isolation and regeneration. Experiments were done to study the effect of several factors such as enzyme concentration, different kinds of osmotic stabilizers and their concentration, mycelial age and mycelial concentration on protoplast release, and the comparison of reversion frequency on media containing different osmotic stabilizers and nutrient components.

(4) Interspecific hybridization between the two Pleurotus species. This included the protoplast fusion and the confirmation of interspecific hybrids obtained.

CHAPTER 2

MATERIALS AND METHODS

A. MATERIALS

I. Fungal Strains

Dikaryotic mycelia of Pleurotus sajor-caju (P127), were obtained from Dr. Zakia Barno, Central Food Technological Research Institute, Mysore, India. Those of P. florida (P110) were provided by Dr. F. Zadrazil, West Germany.

II. MEDIA

(a) Complete Medium (CM) (Raper and Miles, 1958)

Complete medium contained (g per l) $MgSO_4.7H_2O$, 0.5; KH_2PO_4, 0.46; K_2HPO_4, 1.0; Bacto-peptone (Difco), 2.0; D-glucose, 20.0; Bacto-yeast extract (Difco), 2.0; thiamine-HCl, 5 mg.

(b) Minimal Medium (MM) (Vogel, 1964)

Minimal medium contained (g per l) asparagine, 2.0; $MgSO_4.7H_2O$, 0.5; KH_2PC_4, 0.46; K_2HPC_4, 1.0; and glucose, 20.0.

(c) Osmotic Complete Medium

Osmotic complete medium contained complete medium supplemented with 0.6 M of one of the three osmotic stabilizers $MgSO_4.7H_2O$, KCl and mannitol.

(d) Osmotic Minimal Medium

Osmotic minimal medium contained minimal medium supplemented with 0.6 M of one of the three osmotic stabilizers $MgSO_4.7H_2O$, KCl and mannitol.

(e) Agar Media

Solidified agar medium contained 2% Bacto-agar (Difco) and soft top agar medium contained 0.6% Bacto-agar (Difco).

B. METHODS

I. Culture Methods

(a) Incubation conditions

Liquid cultures were incubated on a shaker (Incubator Shaker, New Brunswick Sci. Co.) at 120 rpm . All cultures were incubated at 28°C in the dark unless stated otherwise.

(b) Inoculation

Agar plugs were lifted up with a sterile inoculation knife, from the periphery of the cultures, and put up-side-down onto the test agar plates for subculture.

(c) Agar overlay method

Molten soft agar medium (42°C) was dispensed at a rate of 2.5 ml per tube into test tubes (1.2 cm x 7.5 cm) which were held at 42°C in a constant-temperature steel block (thermoblock). A suitably diluted suspension of 0.1 ml was added to the melted soft agar in the tubes. The tube contents were poured over the bottom agar layer which was rapidly swirled to ensure even distribution before incubation.

(d) Fructification tests

The dikaryons or heterokaryons to be tested were subcultured onto agar medium in plates, in test tubes, or in cotton waste in bottles. When colonies almost covered the whole surface of the medium, the mycelial cultures were ready for induction of primordia. The cultures were then transferred to day light conditions in the laboratory. The first visible sign of the fruiting response was the appearance of tiny hyphal aggregates which were the primordia (Li, 1980). As soon as primordia were formed, the culture plates were placed in polyethylene bags each with four holes (5 mm in diameter) punched on the side 3 cm from the bottom, and the lids of the plates were removed to allow better aeration. Before closing, the bags were filled with 0.5 cm of water to keep the inside

moist. Cultures in bottles were drawn out and placed in moist, light conditions as soon as primordia appeared.

II. Nuclear Staining

Mycelia were subcultured onto sterilized water-permeable cellophane membranes (dialysis tubing, Arthur H. Thomas Co., USA) which were placed on an agar complete medium. After incubation, mycelia grown on the cellophane membranes were fixed in ethanol-acetic acid (3:1) at room temperature for 24 hr. For hydrolysis, mycelia were brought down to water through 95% and 75% ethanol, 30 min each, and treated for 10 min at 60°C with 1 N HCl. After rinsing with water, mycelia were stained in Giemsa solution for 40 - 60 min (Hou and Wu, 1972).

III. Biological Studies

(a) Collection of basidiospores and determination of spore germination

Basidiospores were collected on a sterilized filter paper, in a sterile petri dish (9 cm in diameter), by hanging the fruit body a few cm above the paper until spore prints were formed on the paper. Spore prints were sealed in plastic bags with dry silica gel and stored in the refri-

gerator at 2 - 4 °C until use.

The percentage of spore germination was determined by the following method. A small piece of the filter paper with spore prints was shaken in a small test tube containing 0.5 ml sterilized distilled water. A suspension of 0.1 ml was spread evenly on an agar medium with a sterile bent glass rod and the seeded agar plate was incubated for 48 hr. The percentage of germination was determined microscopically by counting the number of germinating spores out of 100 mature spores (chosen at random). This was done on five sites per plate and the average value was taken (Li, 1980).

(b) Isolation of monokaryons

A spore suspension, prepared as described above, with an appropriate dilution of 500-1000 spores per ml was made. 0.1 ml portions of such suspensions were spread on agar medium by means of a sterile bent glass rod. The seeded agar plates were incubated for 48 hr when the spores started to germinate. Germinating spores were isolated singly by means of a lens-(6x)-equipped cutter and subcultured as above (Eger, 1978).

(c) Determination of mating type of the monokaryotic isolates

For the determination of the mating type of monokaryo-

tic isolates derived from the same fruit body, isolates were tested by being paired in all possible combinations. Inocula of 4 mm^2, cut from the contact margin of mycelial colonies on a week old culture, were transferred onto fresh agar complete medium. Pairings were done by placing two inocula close together and incubated for 6-7 days. All pairings were examined microscopically at the margin of the component mycelial colonies. The mating reaction was recognized as positive when clamp connections were found in the mycelia. When the four classes of mating types were established, one monokaryon of each class was selected and these were used as the mating type testers (Eger, 1978).

(d) Fertility tests with established dikaryons

Small inocula (4 mm^2), cut from the margin of each established dikaryotic colony, were transferred onto test agar plates or to the cotton waste in bottles and incubated. After the mycelia have penetrated through the medium, the cultures were transferred to day light conditions for further development into normal fruit bodies.

(e) Mycelial growth rate

The isolates to be tested were subcultured onto agar plates. After the incubation period, the average diameter (cm) of the colonies for each isolate was

measured and used for comparing their mycelial growth rate.

IV. Induction and Characterization of Auxotrophic Mutants

(a) UV-killing curve

Both basidiospores and mycelia were used for mutant induction. A spore suspension, suitably diluted, was poured onto a sterile petri dish (9 cm in diameter) and exposed to UV (dose of 20 ergs per sec per cm; UV source, 15 W Philip germicidal lamp) with constant agitation using a magnetic stirrer. Samples of the suspension, taken at different time intervals, were suitably diluted before plating onto the complete medium. A non-irradiated sample was used as control. Mycelia from monosporous liquid cultures were macerated in liquid medium at full speed for 2 min in a blender (Waring, commercial blender). 15 ml portions of the mycelial suspensions were transferred to a sterile petri dish (9 cm in diameter) and UV-irradiated as above. The mycelial suspensions were then spread evenly on a complete medium using a sterile bent glass rod. After 4 - 5 days of incubation, colony counts were made.

UV-irradiation and plating were carried out under diffused-light of a red-lamp and the culture plates were kept covered with aluminium foil to prevent

photoreactivation. The number of colonies on culture samples taken at different time intervals during UV-irradiation was recorded, and a survival curve was plotted.

(b) Enrichment of auxotrophs

Mycelia or spores suspended in liquid minimal medium, were UV-treated with a 98% mortality dosage and incubated for 2 hr. Cycloheximide (Sigma) at a concentration of 100 µg per ml was then added. The cultures were incubated for 2 more days, and finally plated onto complete medium after being washed with distilled water. Filtration through glass wool column was also employed as enrichment for auxotrophs when spores were used. Filtration was carried out after UV-irradiated spore suspensions were incubated for 36-48 hr (Burnett, 1975).

(c) Isolation of auxotrophic mutants

For the induction of auxotrophic mutants, spores or macerated mycelia were UV-treated with a 98% mortality dosage. Aliquots of the UV-irradiated materials were then spread uniformly by means of a sterile bent glass rod on a complete medium at a suitable density which would give ca. 50 surviving colonies per plate. These were incubated for 7 - 8 days. Viable colonies developed were isolated singly and transferred onto fresh complete medium.

After incubation, these cultures were used for further experiments.

Small mycelial plugs (4 mm^2) taken from each colony were subcultured onto minimal medium. Isolates which failed to grow were tested for their nutritional requirements on a series of screening media as will be described below. Mutants of P. sajor-caju were designated as Su and that of P. florida as Fu.

(d) Characterization of auxotrophic mutants

Auxotrophic mutants from the above experiments were identified by determining their ability to grow on a series of 12 screening media, according to Holliday's method (1956). The screening media were prepared by supplementing a series of minimal media with different combinations of 6 growth nutrients (Table 2.1). Mycelial growth on complete and minimal media served as control. These auxotrophs were further confirmed by adding the single nutrient required to each minimal medium after the deduction of result from screening media.

The mating type of identified auxotrophs was determined by the pairing of each auxotroph with the four monokaryotic testers.

(e) Isolation of drug-resistant mutants

Drug-resistant mutants were selected by plating the

Table 2.1 A list of components used in Holliday's mixtures*.

	1	2	3	4	5	6
7	adenine	biotin	phenylalanine	alanine	arginine	leucine
8	hypoxanthine	folate	purine	cysteine	ornithine	glycine
9	cytosine	pantothenate	tryptophan	threonine	aspartate	isoleucine
10	guanine	pyridoxin	tyrosine	thiosulphate	proline	histidine
11	thymine	thiamin	aminobenzoate	methionine	glutamate	lysine
12	uracil	riboflavin	nicotinate	choline	inositol	valine

*Each pool contained 1.0 µg per ml of vitamins, 50 µg per ml each of all other compounds.

macerated mycelia at a high density on a complete medium containing a high enough concentration of cycloheximide and chlortetracycline which would inhibit the growth of wild-type mycelia. After incubation, surviving colonies of drug-resistant mutants were singly isolated and subcultured on fresh agar medium .

V. Protoplast Isolation and Reversion

(a) Preparation of mycelia for protoplast isolation

Stock cultures of monokaryons in complete medium were used. Several mycelial inocula (4 mm^2) cut from the stock cultures were subcultured in 10 ml liquid complete medium in 50-ml flasks and incubated for one week. The mycelial colonies in liquid medium were macerated at full speed in a blender (Waring, commercial blender) for 30 sec in fresh liquid complete medium. The mycelial suspensions were transferred into 50 ml flasks containing 10 ml liquid complete medium and incubated for one week.

(b) Preparation of enzyme complex solution

Two kinds of lytic enzymes were used for this experiment: one was Novozym 234 which was supplied by Novo Industri A/S, Denmark; the other was Cellulase CP which was a gift from John and E. Sturge Ltd., Selby, United Kingdom. Both enzymes were stored at -20 °C. The two enzymes

were dissolved in a 0.01 M phosphate buffer (pH 5.8) and the solution was purified by centrifugation 17,369 xg (Sorvall) at 4 °C for one hr in order to remove any contaminants. The supernatant was added to the digestion mixture which contained an osmotic stabilizer.

(c) Preparation of osmotic stabilizer solution

Three chemicals , $MgSO_4.7H_2O$, KCl and mannitol, were used as osmotic stabilizers in the experiments to compare their effectiveness. Solutions at various concentrations, prepared in a 0.01 M phosphate buffer (pH 5.8) were autoclaved at 121 °C for 20 min.

(d) Protoplast isolation

For preliminary tests, 2-day old mycelia, cultured in liquid complete medium were used. Mycelia, recovered aseptically by vacuum filtration and washed twice with 0.6 M $MgSO_4$, were suspended at a final concentration of 20 mg fwt per ml in a digestion mixture of 0.6 M $MgSO_4$ containing 5 mg per ml each of Novozym 234 and Cellulase CP. After incubation, protoplasts were detected microscopically as osmotically sensitive spherical bodies (Hamlyn et al., 1981), and their yields were determined by using a haemocytometer. Five counts were taken for each treatment.

2-mercaptoethanol was used as a pretreatment chemical

prior to the incubation of mycelia in the digestion mixture. 2-mercaptoethanol solutions at various concentrations, prepared in a 0.01 M phosphate buffer containing 0.6 M $MgSO_4$(pH 5.8), were sterilized by filtration through millipores (0.45 um). After 30-min incubation in 2-mercaptoethanol, the mycelia were washed twice by centrifugation at 800 xg for 20 min.

(e) Purification of protoplasts

After 6 hr of incubation in the enzyme complex solution, the mycelial debris was removed by filtration through a column of sterile glass wool (about 4 - 5 cm in length). The protoplasts in the filtrate were collected by centrifugation at 7,000 x g for 40 min. The protoplast pellet was washed twice with a 0.6 M osmotic stabilizer solution and resuspended in fresh osmotic solution.

(f) Detection of protoplast reversion

For the observation of protoplast reversion at the primary stage, purified protoplasts were suspended in 3 ml of liquid complete medium supplemented with 0.6 M mannitol in 25-ml flasks. During incubation, regenerating protoplasts were transferred, at intervals, by means of a sterile pipette onto glass slides. The morphology of these protoplasts was observed under a phase contrast microscope.

(g) Reversion frequency of protoplasts

Purified protoplasts, obtained by the above method, were diluted appropriately with 0.6 M $MgSO_4$ in a 0.01 M phosphate buffer at pH 5.8. An aliquot of 0.1 ml was added to a soft agar minimal or complete medium (42°C), containing 0.6 M $MgSO_4$, KCl or mannitol, and plated by means of agar overlay method. The plates were incubated for 5 - 6 days. Reversion frequency was determined by the ratio of the number of colonies formed per plate to the number of protoplasts plated.

VI. Interspecific Protoplast Fusion

(a) Protoplast fusion

Purified protoplasts were suspended in 0.6 M mannitol and the concentration of the protoplasts was determined with a haemocytometer. The protoplast mixture was prepared by adding 5×10^6 protoplasts of each parental strain to a sterile screw-capped centrifugation tube. The mixture was centrifuged as described above, and the protoplast pellet was resuspended in 1 ml 30% PEG in 0.1 M $CaCl_2$ and 0.05 M glycine (pH 7.5 , 30°C). After incubation for 10 min at 30 °C, the suspension was diluted with 4 ml of 0.6 M mannitol. Replicated platings of protoplasts on osmotic minimal medium were made with 0. 1 ml

undiluted suspension, and 0.1 ml and 0.5 ml of 10 times diluted suspension. Platings on osmotic complete medium were made with 0.1 ml and 0.5 ml of 10 times diluted suspensions. Replicate platings were set up. After 7 - 9 days of incubation, the cultures were checked for regeneration and colony formation. Complementation frequency was calculated according to the following formula:

$$\frac{\text{Number of colonies on osmotic minimal medium}}{\text{Number of colonies on osmotic complete medium}} \times 100$$

Several control experiments were set up as follows:

(1) Mixtures of protoplasts of the two parental strains without PEG treatment;

(2) Mixtures of lysed protoplasts;

(3) Protoplasts of the parental strains treated with PEG and plated separately.

(b) Isolation of interspecific heterokaryons

When the colonies which had regenerated on minimal medium were about 0.5 cm in diameter, they were isolated and subcultured on agar minimal medium in test tubes and on minimal agar plates. After the mycelial colonies occupied the whole surface in the test tubes, they were kept at 4 °C as stock cultures. Mycelia grown on minimal medium were used for further studies such as

comparison of growth rate on complete and minimal media, investigation of nuclear number per cell, mating reactions, fructification and biochemical analysis.

(c) Preparation of polyacrylamide gel for electrophoresis

The preparation of polyacrylamide gels was according to the method adopted by Davis (1964). Gels for isoenzyme electrophoresis were prepared from the stock solutions listed in Table 2.2. The running (lower) gel consisted of 1 part of solution A, 2 parts of solution C, 4 parts of solution F, and 1 part of water. The spacer (upper) gel contained 1 part of solution B, 2 parts of solution D, 1 part of solution E, and 4 parts of water.

For the preparation of gel columns, glass tubes (10 x 1 cm internal diameter) were tightly wrapped at one end with parafilm (American Can Company) and arranged upright on a rack. The running gel solution, added at the rate of 5.5 ml per tube, was covered with 0.1 ml of distilled water. After photopolymerization under a daylight fluorescent bulb for about 30 - 45 min, the water layer was removed and replaced with 1.5 ml of spacer gel solution. After overlaying the spacer gel solution with 0.1 ml of distilled water, the tubes were again placed under a fluorescent light for photopolymerization.

Table 2.2 Stock solutions used for gel electrophoresis.

(A)	Tris	36.6	g	
	TEMED	0.23	ml	
	1N HCl	48.0	ml	
	H_2O	to 100.0	ml	(final pH 8.9)
(B)	Tris	5.98	g	
	TEMED	0.46	ml	
	1N HCl	48.0	ml	
	H_2O	to 100.0	ml	(final pH 6.7)
(C)	Acrylamide	28.0	g	
	Bis	0.74	g	
	H_2O	to 100.0	ml	
(D)	Acrylamide	10.0	g	
	Bis	2.50	g	
	H_2O	to 100.0	ml	
(E)	Riboflavin	4.0	mg	
	H_2O	100.0	ml	
(F)	Ammonium persulphate	0.14	g	
	H_2O	to 100.0	ml	

(d) Extraction of isoenzyme from mycelia

Mycelia were homogenized in a blender (Waring commercial blender) in a 0.1 M phosphate buffer at pH 6.0 (0.5 g fwt mycelia per ml buffer) at 4 °C. The homogenate was then centrifuged at 7,719 xg (Sorvall), for 20 min at 4 °C. The supernatant was assayed for electrophoresis.

(e) Electrophoresis

A sample of 1.0 ml isoenzyme solution was mixed with 0.1 ml of 0.05% bromophenol blue which served as a tracking dye. By means of a microsyringe, a sample solution of 100 µl was applied to the top of the spacer gel. An electrode buffer consisted of 6.0 g Tris (Sigma) and 28.8 g glycine (Sigma) in 1 l distilled water was used as the stock buffer solution. The stock buffer solution was diluted to 1/10 strength with distilled water before use. With the anode connected to the bottom reservoir and the cathode to the top reservoir, electrophoresis was run at a constant current of 1.5 mA per gel column at 4 °C until the bromophenol blue moved to within 1 cm of the gel bottom.

(f) Gel staining

The following staining methods, carried out at room temperature, for different isoenzymes were described by Wetter

(1982):

Peroxidase: Isoperoxidases were located by staining the gel with a solution consisting of 5 mM H_2O_2 (freshly prepared) and 10 mM guaiacol in phosphate buffer (10 mM, pH 7.0);

Esterase: The staining solution contained 20 mg of a-naphthyl acetate (Sigma) and 3.8 mg of Fast Blue RR salt (Sigma) in 50 ml of 0.2 M phosphate buffer at pH 6.0;

Alcohol dehydrogenase (ADH): The staining solution contained 40 mg NAD (Sigma), 1 mg phenazine methosulphate (Sigma) and 20 mg nitroblue tetrazolium (Sigma) in 46 ml of 0.1 M Tris buffer at pH 8.5. Immediately before staining, 0.5 ml of 95% alcohol was added into 10 ml of above solution. The staining procedure was carried out under dark condition.

The relative mobility, Rf, is defined as the mobility of the isoenzyme(s) measured with reference to the tracking dye and calculated by the formula:

$$Rf = \frac{\text{Distance migrated by protein}}{\text{Distance migrated by dye}}$$

CHAPTER 3

BIOLOGY OF *PLEUROTUS SAJOR-CAJU* AND *P. FLORIDA*

A. INTRODUCTION

I. Sexuality in Basidiomycetes

The majority of cultivated mushrooms belong to the highly evolved fungal group, the basidiomycetes. Up to now, sexuality of many cultivated mushrooms has been understood from studies on the fertility patterns in matings among the progeny, on the cytology throughout the life cycle, and on genetic analysis. In general, the life cycle of Basidiomycetes can be divided into three distinct nuclear phases: the haploid phase, the heterokaryotic dikaryon phase, and the transient diploid phase. A homokaryon which is a mycelium derived from a single spore, usually has uninucleate cells and is known as a monokaryon. A heterokaryon is formed by the hyphal fusion (plasmogamy) between two genetically distinct homokaryotic mycelia of compatible mating types.

The common pattern of sexuality in basidiomycetes is heterothallism (Raper, 1978), a term which is used to define the breeding system of fungi which are self-incompatible and cross-compatible. Therefore, plasmogamy,

in cross-mating between two different homokaryotic mycelia, is an essential process for fulfilling the life cycle for a heterothallic species. The sexuality of heterothallism is controlled by the genetic factors of the incompatibility systems. There are two types of incompatibility systems: (1) the unifactorial or bipolar system in which sexuality is controlled by a single genetic factor; and (2) the bifactorial or tetrapolar system in which sexuality is controlled by two unlinked genetic factors, A and B. In the tetrapolar system, the two homokaryons must differ with respect to their A factors as well as to their B factors in order to develop a fertile dikaryon.

II. Mating Reactions in Basidiomycetes

As reviewed by Raper (1966), the occurrence of tetrapolar incompatibility is determined by paired factors of two series, A and B, which segregate and assort independently at meiosis. In other words, four mating types of single—spore isolates are formed with two different A and two different B factors, e.g., AxBx, AxBy, AyBx, and AyBy, from a single fruit body. Thus, there is no dominance-recessivity relationship in such two-allele systems. A spore of each type develops into a self-sterile but cross-fertile homokaryon. After hyphal

fusion, the A and B incompatibility factors control the distinctiveness by coordinating the different parts of the mating reactions. The detail of its operation has been studied most comprehensively in Schizophyllum commune (Raper and Raper, 1966). When a homokaryon from one type was paired in mating with that of each of the other three types, three distinct patterns of morphological events occurred. When the A incompatibility factors were different but the B's were the same (e.g., A≠B=), only part of the series of events (A- on) occurred. This interaction induced the pairing of parental nuclei in a cell, the formation of a hook cell at each septum and the conjugate nuclear division. When the B's were different but the A's were the same (A=B≠), the B- sequence occurred and involved the reciprocal exchange and the migration of the nuclei throughout the mycelium of each mate with the formation of clamp connections. The entire progression of sexuality leading to the development of a fertile dikaryon could only be induced by different alleles in both A and B factors (A≠B≠). Moreover, both A and B factor are constituted of two linked genes, α and β, and each gene has multiple states with respect to specificity. There has been no evidence that the products of α and β genes witnin a factor interact within each other but they appear as functional equivalents (Raper, 1978).

The structure and function of incompatibility

factors regulating the sexual development in mushrooms has become apparent from the variety of mutations obtained in the incompatibility loci. Various mutants have been isolated in the B_β gene that range in effects from dominant (always-on) through several intermediate intensity of changes in specificity and function, to recessive (always-off) (Raper and Raper, 1973). These mutants which demonstrated the controlling functions of B incompatibility factor, included self versus nonself recognition in the allelic interaction, the nuclear migration, and the fusion of hook cells. Furthermore, some mutants obtained in this single incompatibility locus confirmed that this complex factor did consist of two major parts, one for specificity concerned with allelic specificity (i.e. self-recognition), and the other for the function of initiating and regulating the B- sequence of sexual morphogenesis (Raudaskosky et al., 1976). Raper and Raper (1973) also suggested that the B gene operated via a positive control mechanism and involved at least four functions: (1) specificity, (2) nuclear migration, (3) nuclear exchange, and (4) fusion of hook cell.

III. Incompatibility System and Taxonomy in *Pleurotus*

The genus *Pleurotus* belongs to the family Polyporaceae (Fr.) of Hymenomycetes. According to

Singer (1975), this genus consists of a total of 39 species which can be divided into five sections which are Lepiotarii, Pleurotus, Calyptrati, Lentodiallum and Tuber-Regium. Among the species, P. ostreatus has been studied in most details. In this species, the tetrapolar pattern has been clearly demonstrated by the analysis of clamp connection formation in a large sample of sibling pairings (Vandendries, 1933), and its bifactorial heterothallism has been confirmed by several workers (Terakawa, 1957; Eugenio and Anderson, 1968). It was found that each fruit body produced basidiospores of four mating types with equal frequencies due to the independent assortment of the incompatibility factors (A and B) in meiosis. The tetrapolar mating system was also observed in other Pleurotus species including P. sapidus (Eugenio and Anderson, 1968), P. flabellatus (Yusof and Graham, 1977) and P. sajor-caju (Roxon and Jong, 1977). There is still much confusion in the classification of this genus Pleurotus (Eger et al., 1976; Kurtzmun and Zadrazil, 1982; Eger et al., 1979). In most cases, species delimitation based on morphological differences may be completely wrong as it is especially true in fungi. In view of this, it has been proved that single gene mutations could cause gross alterations of the phenotype without having any change on the mating capacity of the organism concerned (Esser and Kuenen,

1967). Eger and co-workers (1979) found that, within the P. ostreatus species complex, it was very difficult to identify an isolate on the basis of morphological characteristics including macroscopic and microscopic phenotypes. Hence, they suggested that the best way to avoid such a confusion in the taxonomy of mushrooms could be by means of mating tests among the strains in question.

During the last few years, mushroom growers have become more interested in the commercial cultivation of P. ostreatus (oyster mushroom). It was estimated that Pleurotus, together with Agaricus, Lentinus and Volvariella are, at present, the four most important cultivated edible mushrooms (Delcair, 1978). The simple and oldest method to grow oyster mushroom is to use wooden blocks cut from tree trunks as the substrate, while in the modern culture techniques, agricultural waste materials are used as the substrate. Cultivation experiments of Pleurotus have now been carried out all over the world. P. ostreatus can grow well on various kinds of sawdust and other natural organic material (Block et al., 1959). One species, often called Pleurotus "Florida", isolated in the subtropical Florida in US by Block et al. (1958), was found to grow best at 25 - 30°C (Zadrazil, 1976). The effect of environmental factors on the cultivation of P. sajor-caju, isolated in India, was

reported by Jandaik and Kapoor (1976). The optimum fruiting temperature was around 25°C and the maximum 30°C, and the presence or absence of light did not affect the development of fruit bodies. Based on the temperature requirement for fructification, P. sajor-caju should be the most promising species for tropical and subtropical regions. It was noticed that P. sajor-caju, when grown on cotton waste, had higher protein and comparable carbohydrate contents when compared to that of Agaricus bisporus, Lentinus edodes, Pleurotus ostreatus and Volvariella volvacea (Chang et al., 1981).

B. RESULTS

I. Morphological Differences Between the Two Species

After subculture, mycelia could completely permeate the cotton waste medium within 2 weeks at 25°C in the dark. The formation of fruit bodies of both species required 10 - 14 more days after cultures were transferred to light conditions.

In dark conditions, P. florida formed no fruit bodies (sporophores), while P. sajor-caju could form fruit bodies easily on the agar complete medium but they produced primordia in clusters and the fruit bodies were more or less abnormal. In light conditions, both species could

form fruit bodies. The morphology and colour of the fruit bodies were quite different between these two species (Fig. 3.1). The fruit bodies of P. sajor-caju grew individually on the cotton waste and their colour was grey. In contrast, the fruit bodies of P. florida grew in clusters and were white and yellow in colour. In heavy deposits, spore prints of P. sajor-caju were grey and those of P. florida were yellow in colour.

The vegetative growth of both species was rapid on the agar complete medium (about 5 - 6 cm in diameter after the first week). Mycelial growth of P. florida was more luxuriant, and was white in colour during its initial growing stage (Fig. 3.2). Microscopically, the mycelium of P. florida was found to have more branches which were longer than that of P. sajor-caju at the main hyphal apical region (Fig. 3.3).

II. Isolation of Monokaryons and Mating Reactions in P. sajor-caju

Monokaryons of P. sajor-caju were isolated from the germlings of a single fruit body. After these were mating-tested, nine single-spore isolates were obtained from a single fruit body. For the determination of mating type, the single-spore isolates were paired in all possible combinations. The preliminary results are shown

Fig. 3.1 The morphology of Pleurotus fruit bodies from one-month cultures on cotton waste at 28 °C.

(a) P. sajor-caju (P127).

(b) P. florida (P110).

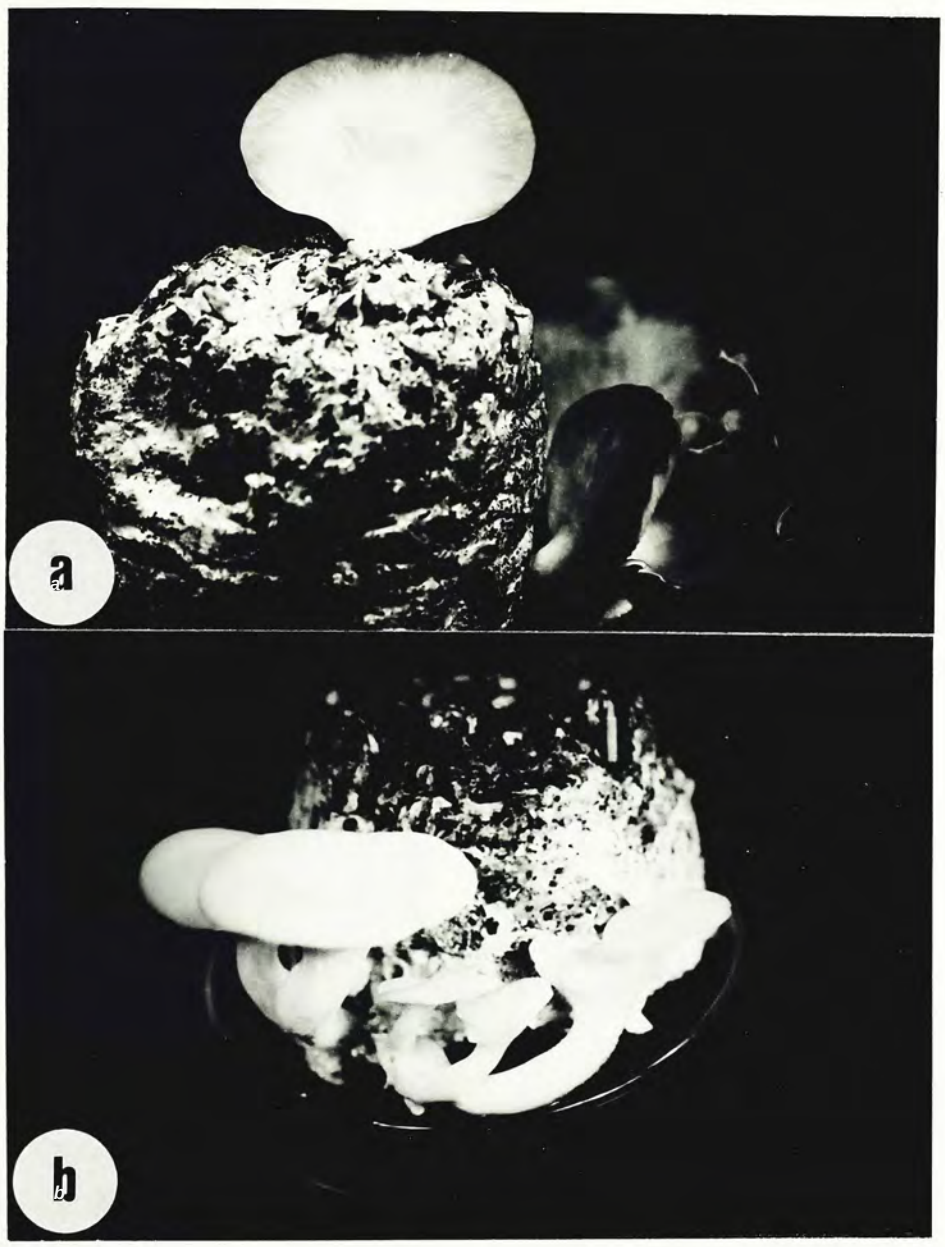

a
b

Fig. 3.2 Comparison of mycelial colony morphology between the monokaryotic testers from Pleurotus sajor-caju (left) and from P. florida (right).

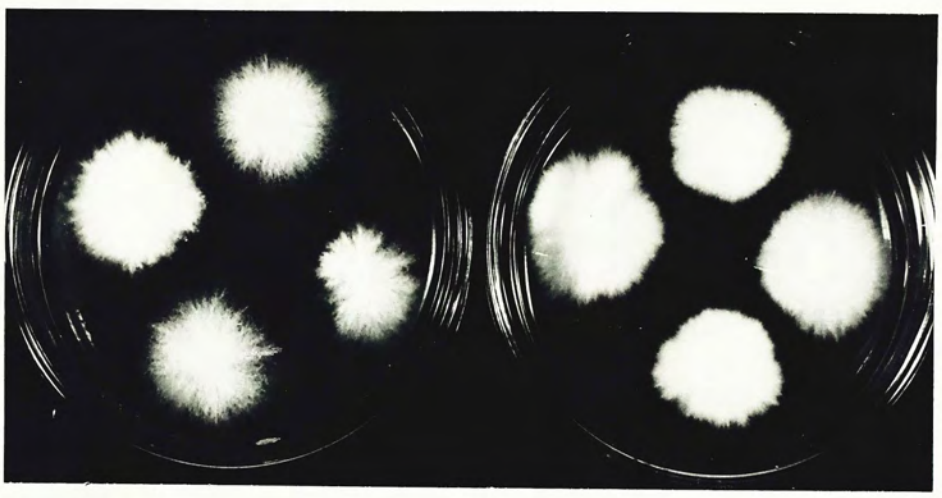
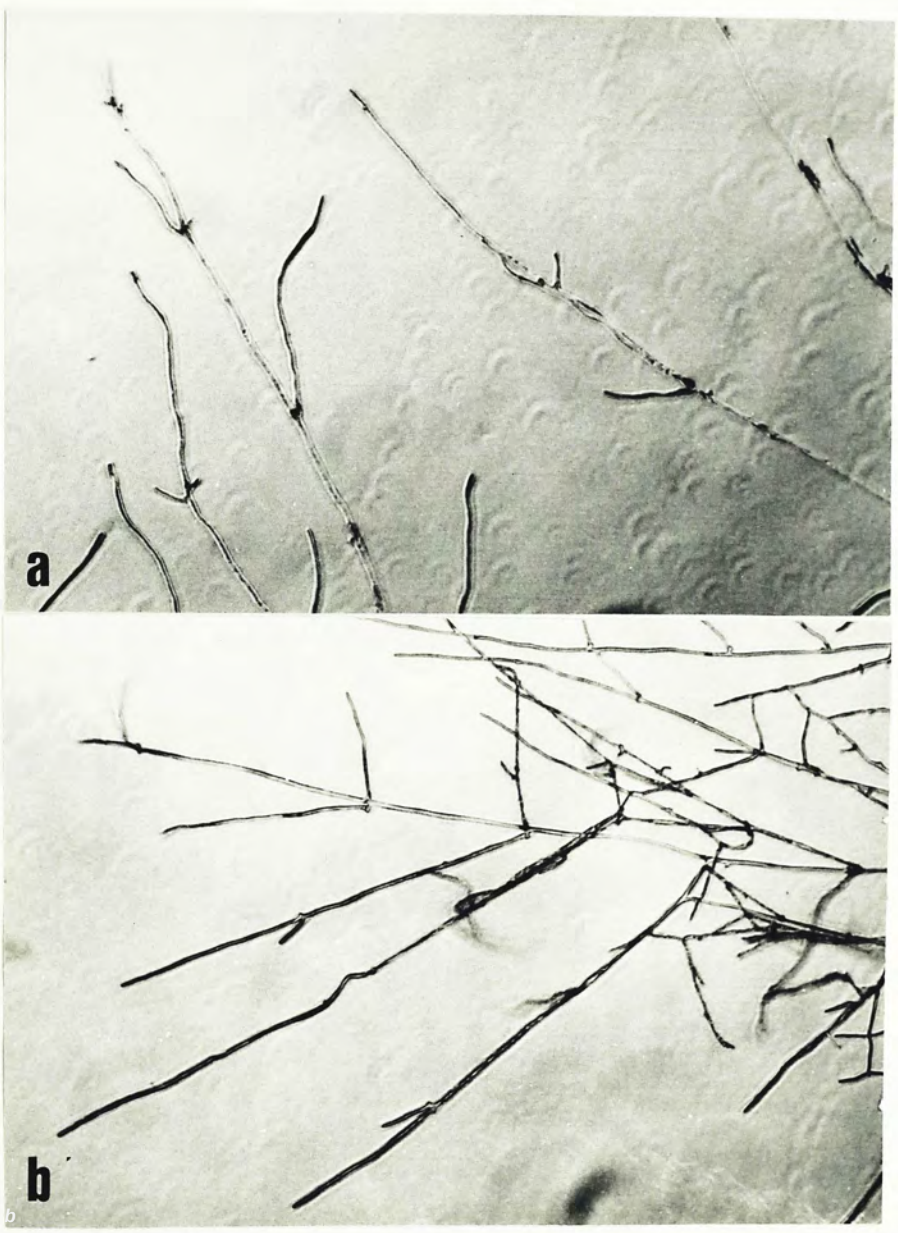


Fig. 3.3 The morphology of mycelia of Pleurotus.

Magnification 100x.

(a) P. sajor-caju (P127).

(b) P. florida (P110).


a
b

in Table 3.1, where "+" means dikaryotisation and "-" means no dikaryotization. Dikaryotization was determined by the formation of clamp connections which were usually located at the branch points of single hyphae at the margin of the colonies (Fig. 3.4). The results obtained from these matings could be represented as shown in Table 3.2. The monokaryons of isolates S9 and S36 were compatible with those of nos. S23 and S24, and thus, were designated as uncompatibility classes 'I' and 'II' respectively. The monokaryons of isolates S22 and S26 and those of isolate S27 were compatible and were designated as classes 'III' and 'IV' respectively. As expected, the monokaryons from a single fruit body could be assigned into these four mating type classes. Using these four tester strains, other single-spore isolates including the mutants from the same fruit body could be grouped accordingly.

Figure 3.5 shows the mating reactions when monokaryons of one tester (S22) were paired with those of each of the other three mating type testers. There was no clear external distinctiveness among the four mating reactions observed during the mycelial growth.

III. Isolation of Monokaryons and Mating Reactions in *P. florida*

Single-spore isolates of *P. florida* were mated

Table 3.1 Mating reaction among monokaryotic strains (S) from Pleurotus sajor-caju

(+, dikaryotization; -, no dikaryotization.)

Monokaryotic strain	S9	S22	S23	S24	S26	S27	S36
S9	-						
S22	-	-					
S23	+	-	-				
S24	+	-	-	-			
S26	-	-	-	-	-		
S27	-	+	-	-	-	-	
S36	-	-	+	+	-	-	-

Fig. 3.4 Formation of clamp connections (indicated by arrows) on the dikaryotic mycelia of Pleurotus sajor-caju (P127). Magnification 400X, phase contrast.

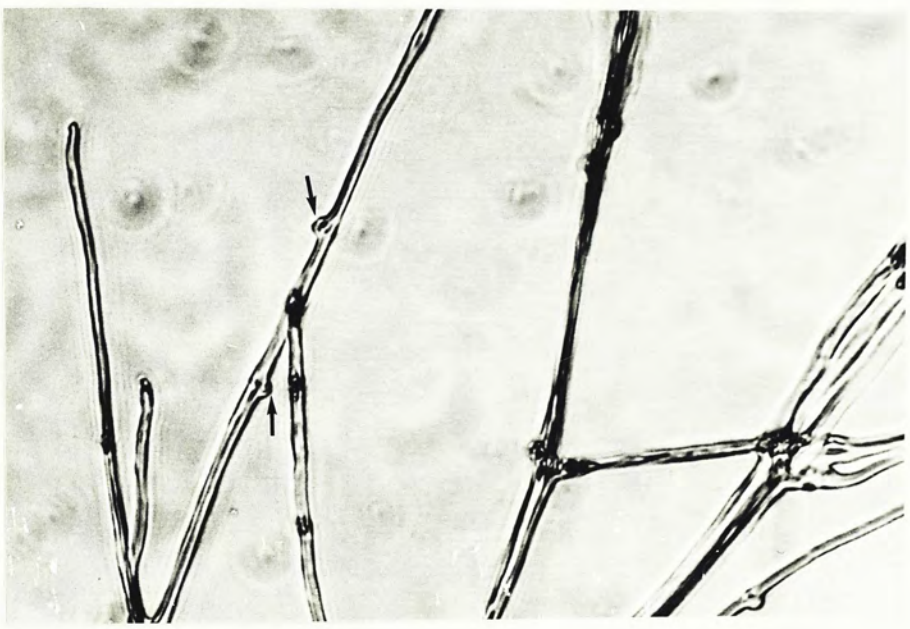


Table 3.2 The resorting classes of mating types among monokaryotic isolates (S) from Pleurotus sajor-caju. (+, dikaryotization; -, no dikaryotization.)

	Mating type						
	I		II		III		IV
Monokaryotic strain	S9	S36	S23	S24	S22	S26	S27
S9	-	-	+	+	-	-	-
S36	-	-	+	+	-	-	-
S23	+	+	-	-	-	-	-
S24	+	+	-	-	-	-	-
S22	-	-	-	-	-	-	+
S26	-	-	-	-	-	-	+
S27	-	-	-	-	+	+	-

Fig. 3.5 Resulting colonies showing mating reactions among the pairings of monokaryotic isolates (S22, S23, S27 and S36) from Pleurotus sajor-caju. Clockwise from top: S22xS22, S22xS27, S22xS23, and S22xS36.

with each other for the detection of clamp connections. The result of dikaryotization among these monokaryons is shown in Table 3.3. As in P. sajor-caju, these monokaryons could also be assigned into four classes of mating types (Table 3.4), and the mating reactions among the monokaryons of these four testers are shown in Figure 3.6. Again, there was no difference in the interaction phenomenon among the pairing reactions.

IV. Incompatibilty Tests between P. sajor-caju and P. florida

For studying the incompatibility between these two species, monokaryons of the four testers of P. sajor-caju were paired with that of P. florida (Table 3.5). Under microscopic observation, there appeared to be no compatible mating in these crosses since no clamp connections were detected on the periphery of any of the colonies. Mating reactions between monokaryons of P. sajor-caju and those of the four mating type testers of P. florida and their reciprocal crosses are shown in Figures 3.7 and 3.8. There was a clear contact zone between the paired mycelial inocula. The monokaryotic mycelial colonies of P. sajor-caju could be distinguished from that of P. florida and the intrusion of mycelial growth into the opposite zone did not occur.

Table 3.3 Mating reactions among monokaryotic strains (F) from Pleurotus florida.

(+, dikaryotization; -, no dikaryotization.)

Monokaryotic strain	F1	F2	F6	F7	F9	F11	F12	F13	F22
F1	-								
F2	-	-							
F6	+	-	-						
F7	+	-	-	-					
F9	-	-	+	+	-				
F11	-	+	-	-	-	-			
F12	-	+	-	-	-	-	-		
F13	-	-	-	-	-	+	+	-	
F22	-	+	-	-	-	-	-	+	-

Table 3.4 The resorting classes of mating types among monokaryotic strains (F) from *Pleurotus florida* (+, dikaryotization; -, no dikaryotization.)

	Mating type								
	I		II		III		IV		
Monokaryotic strain	F1	F9	F6	F7	F2	F13	F11	F12	F22
F1	-	-	+	+	-	-	-	-	-
F9	-	-	+	+	-	-	-	-	-
F6	+	+	-	-	-	-	-	-	-
F7	+	+	-	-	-	-	-	-	-
F2	-	-	-	-	-	-	+	+	+
F13	-	-	-	-	-	-	+	+	+
F11	-	-	-	-	+	+	-	-	-
F12	-	-	-	-	+	+	-	-	-
F22	-	-	-	-	+	+	-	-	-

Fig. 3.6 Resulting colonies showing mating reaction among the pairings of monokaryotic isolates (F1, F6, F12, and F13) from Pleurotus florida. Clockwise from top: F1xF1, F1xF6, F1xF12, and F1xF13.

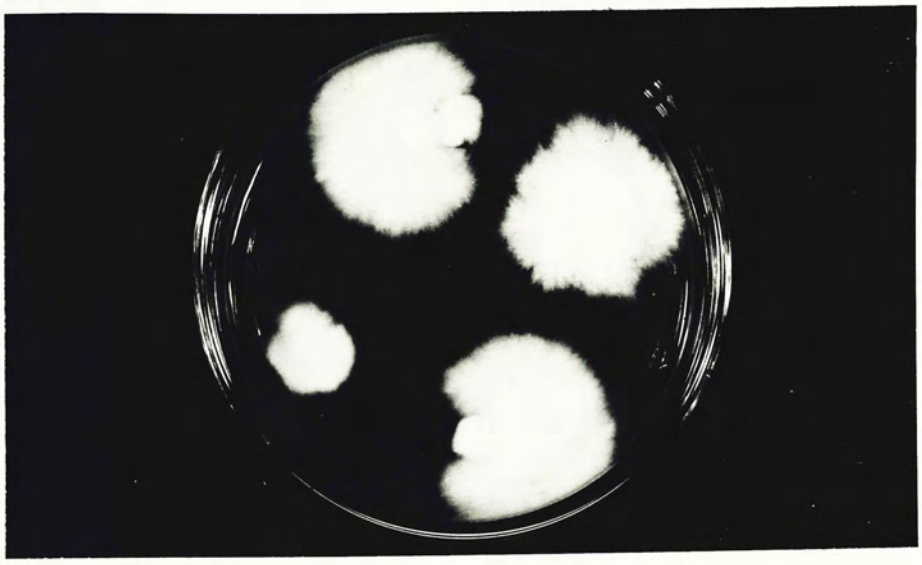


Table 3.5 Mating reactions among monokaryotic strains (F) from Pleurotus florida and monokaryotic strains (S) of four mating types from P. sajor-caju. (-, no dikaryotization.)

P. florida Monokaryon		P. sajor-caju Monokaryotic taster			
Mating type	Strain	S22	S23	S27	S36
I	F1	-	-	-	-
	F9	-	-	-	-
II	F6	-	-	-	-
	F7	-	-	-	-
III	F2	-	-	-	-
	F13	-	-	-	-
IV	F11	-	-	-	-
	F12	-	-	-	-
	F22	-	-	-	-

Fig. 3.7 Resulting colonies showing mating reactions among the pairings of monokaryotic strain (S22) from Pleurotus sajor-caju and monokaryotic strains (F1, F6, F12 and F13) from P. florida. Clockwise from top: S22xF12, S22xF6, S22xF13, and S22xF1.

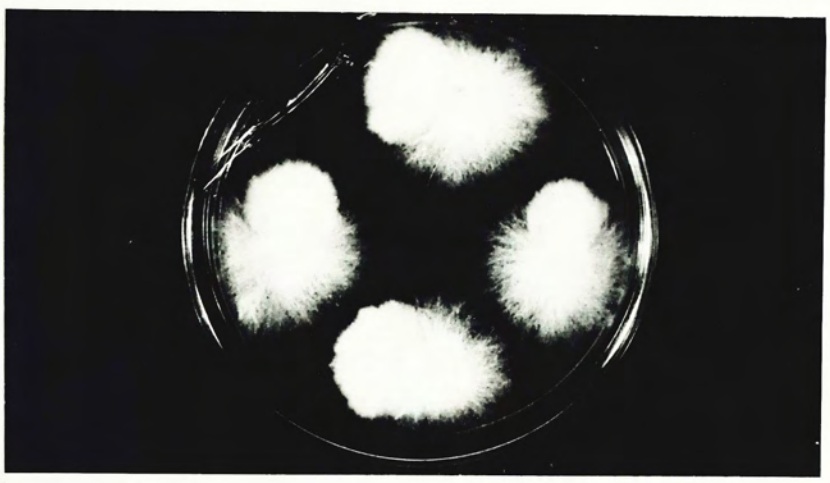
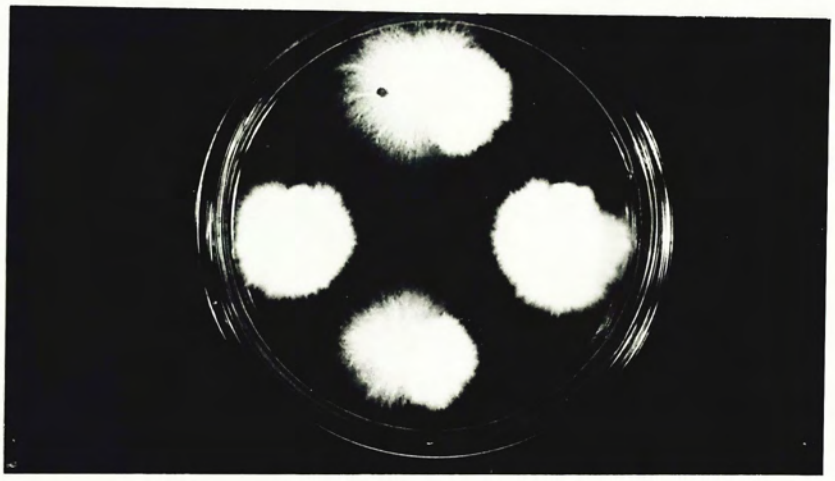


Fig. 3.8 Resulting colonies showing mating reactions among the pairingsof monokaryotic strain (F1) from Pleurotus florida and monokaryotic strains (S22, S23, S27 and S36) from P. sajor-caju. Clockwise from top: F1xS22, F1xS36, F1xS23 and F1xS27.



Furthermore, when inocula of 4 mm^2 from the contact zone were subcultured on fresh agar medium, the contact zone appeared persistently during the mycelial growth but there was still no development of clamped hyphae on the periphery of any of the subcultured colonies.

By means of nuclear staining ,it was noted that the majority of the cells at hyphal tips and subapical regions contained only one nucleus (Fig. 3.9 and Table 3.6). These data show that there was no hyphal fusion in the mating tests between monokaryons of P. sajor-caju and P. florida.

C. DISCUSSION AND CONCLUSION

In recent years, P. florida has been cultivated by mushroom growers throughout Europe and America because of its ease of cultivation and its tolerance of higher temperature when compared to that of P. ostreatus (Eger et al., 1976). However, the taxonomy of P. florida has been disputed and this mushroom was considered by many workers as a subspecies of P. ostreatus on the evidence of mating tests (Block et al., 1959; Eger et al., 1976; Eger et al., 1979). Classification, which includes describing, naming and placing organisms in their proper relationship to one another, is of great importance to the cultivation of mushrooms. It can aid in the evaluation of

Fig. 3.9 Uninucleate cells (indicated by arrows) in mycelia isolated from the contact zone in colonies resulted from mating between Pleurotus sajor-caju strain (S22) and P. florida strains (F1 and F11). Nuclei stained with Giemsa; magnification 1,000x.

(a) S22 x F1.

(b) S22 x F11.

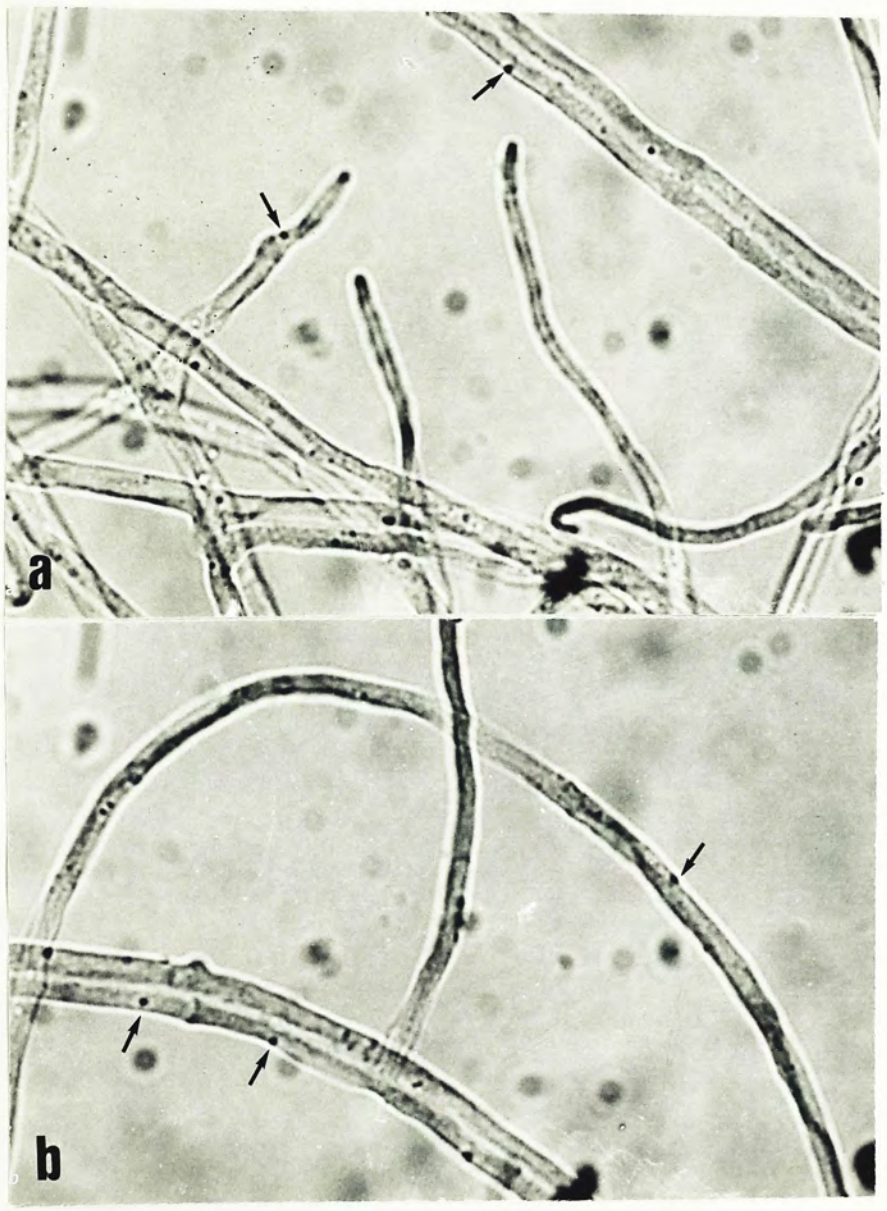

a
b

Table 3.6 Number of nuclei in apical cells and subapical cells of mycelia which were subcultured from the contact zones.

Mating	Apical cell		Subapical cell	
	1	2	1	2
S22 x F1	21	2	50	4
S22 x F6	24	4	21	0
S22 x F11	12	3	42	0
S22 x F13	18	2	31	1
F1 x S22	19	1	47	3
F1 x S23	11	2	37	0
F1 x S27	26	0	29	0
F1 x S36	15	0	23	0

new cultivation methods when compared to those already being used. Furthermore, proper classification also contributes to the usefulness in breeding programmes in which desired objectives have been realized. In general, ecological habitat and morphological characteristics including the colour of sporophores, gills and spore prints and spore form (the ratio of length to diameter) are often used as criteria for the classification in mushrooms (Han et al., 1974; Eger et al., 1979). However, many macroscopic and microscopic characteristics are rather unreliable. It is generally accepted that two strains belong to the same species if interbreeding and the production of viable offsprings can be demonstrated. Based on this delimitation, European P. ostreatus and the American strain "Florida" were regarded as a single species (Eger et al., 1976). Matings of monokaryons isolated from 'German' P. ostreatus and American "Florida" Pleurotus produced hybrid dikaryons with normal growth rate and many of them could develop normal fruit bodies with basidiospores that were able to germinate. Moreover, when these two mushroom strains were cultured under identical condition at low temperature (15°C), no difference in their morphology was observed. The only major difference was that P. florida could fruit well up to 24°C.

Furthermore, European P. ostreatus, P. pulmonarius,

P. columbinus, and American P. sapidus were demonstrated to belong to the same species by mating tests. All these Pleurotus species not only form dikaryons with each other but also produced viable spores and had an incompatibility system in common (Eger et al., 1979). On the other hand, the sporophore morphology of P. sajor-caju was quite different from that of P. florida, including colour and growth habit. Moreover, P. sajor-caju could form fruit bodies easily on agar complete medium even in dark conditions, while P. florida did not form fruit bodies in the dark. Production of fruit bodies of P. sajor-caju in dark conditions has also been reported (Jandaik and Kapoor, 1976). Both species were demonstrated to be heterothallic and tetrapolar as had been previously reported on many Pleurotus species (Vandendries, 1933).

In each mating between two monokaryons of the same fruit body, the characteristics of the resulting heterokaryons can be determined by the specific combination of incompatibility factors carried by the two mates. Four basic types of matings and heterokaryons occur in such tetrapolar species: (a) noncompatible mating with the same A and the same B factors (A=B=), (b) hemicompatible -A mating with like A factor and unlike B facator (A=B≠); (c) hemicompatible -B with unlike A factor and like B factor (A≠B=); and (d) compatible mating with

unlike A and unlike B factor (A≠B≠).

In Schizophyllum commune, the interactions of the four mating types could be recognized in the pairings of the tester strains (Papazian, 1949, 1950 and 1951). It was found that A=B≠ reactions were easily detected by the paucity of aerial hyphae and the appearance of the colony which was termed "flat" and A≠B= with the presence of the "barrage" region along the line of mycelial contact which was limited to the line of confrontation. The common AB heterokaryon (A=B=) showed a gross morphologically distinctive reaction and the different AB heterokaryons (A≠B≠) often had primordial formation on the mycelial colony. Moreover, the distinctiveness of the four mating reactions varies widely in different species. In many cases, the only reaction that is easily recognizable is dikaryotization. For example, neither the A=B≠ nor the A≠B= reaction was macroscopically detectable in Coprinus lagopus (Swiezynski and Day, 1960). In such a case, microscopic examination for clamp connections is definitely necessary.

Vandendries (1933) first reported that P. ostreatus had a tetrapolar mating system. He used the terminology such as a,a' and b,b', for the incompatibility factors. He found that the barrage phenomenon occurred in the common a or common a' mating (e.g. ab + ab', a'b + a'b'). According to Raper (1978), incompatibility factors a,a'

and b,b' correspond to B1,B2 and A1,A2 respectively. Furthermore, Eugenio and Anderson (1968) found that false or incomplete clamps appeared in the barrage region of common B pairings but there were no distinctive characteristics in the common A interaction in P. ostreatus. In P. sajor-caju pseudo-clamps and barrage interaction were only occasionally formed in common B pairings (Roxon and Jong, 1977). As concluded by Eger (1978), barrages are unreliable in P. ostreatus because they can be absent in the common B- and sometimes can only be detected in common A-interaction. Therefore, microscopic observations of clamp connections are definitely necessary in Pleurotus and the grouping of single spore isolates from P. sajor-caju and P. florida in this experiment into incompatibility classes was according to clamp formation. However, clamp connections could not be observed in the interspecific matings between monokaryons of P. florida and P. sajor-caju. In contrast, there was a clear contact zone between the two paired mycelial colonies.

The incompatibility systems and sexuality of the wild four-spore Agaricus species. A. bitorquis, A. nivescens, A. macrosporus and A. silvicola were investigated in detail and compared with that of the two-spore commercially cultivated mushroom, A. bisporus by Elliott (1978). Among the interspecific matings, only

the mating between A. bisporus and A. macrosporus was weakly positive on complete yeast extract medium and there was a zone of inhibited growth between the pairing isolates. All other reciprocal matings among these Agaricus species were found to be negative and many had a demarcation zone in the contact zone. The growth of adjacent isolates tended to overlap in the mating between A. bitorquis and A. nivescens. In the mating of A. bisporus and A. nivescens, the demarcation zone was not observed. However, A. macrosporus and A. nivescens could be considered as the same species, although fruiting was limited to the junction zone between compatible isolates. Kemp (1975) also suggested that microscopic examination for the presence of hyphal fusion and cytoplasmic lethality could help in determining the relationships of the species. Therefore, P. sajor-caju and P. florida described here were distinct on the basis of fruit body characteristics, colony morphology, mycelial interactions and nuclear number per cell in the matings.

CHAPTER 4

THE INDUCTION AND CHARACTERIZATION OF MUTANTS

A. INTRODUCTION

The development of fungal genetics has been almost all based on the study of mutants at specific chromosomal loci (Fincham and Day, 1965). Mutation is defined as an inheritable change in a chromosome (Watson, 1975) and it may modify genes which are generally stable. Mutation takes place in all living organisms and is an important origin of hereditary variations. The capacity to mutate is a property of the genetic material and is as important as stability. A mutation may correspond to the change of a single nucleotide unit in a DNA strand. Mutant genes are employed as markers whereby the genetics of an organism can be investigated. In short, the loci could never be identified nor their function be studied without mutation. For example, the control mechanisms for many developmental processes such as fructification (Stahl and Esser, 1976; Mainhardt and Esser, 1981) and sexuality/incompatibility systems (Raper and Raper, 1973) could be demonstrated by the

mutations of corresponding functional genes which distinguish them from the standard form of wild-type organisms. Biochemical and genetic analysis of mutant strains is also a powerful technique for the study of cellular processes at the molecular level. Moreover, selection following mutagenesis was one of the methods for yeast strain improvement (Spencer and Spencer, 1983). The mutants were used as markers for the recovery of hybrids in the selection procedures of breeding (Cove, 1979; Peberdy, 1980). In fact, a group of biochemical mutants which has not often been studied genetically, is of economic importance for increasing the yield of certain metabolites. Some mutants have been obtained for high penicillin production by Penicillum chrysogenum and for citric acid production by Aspergillus niger (Burnett, 1975).

A convenient distinction can be made between forward and reverse mutations. Forward mutations are those from the wild-type genotypes to non-wild or mutant genotypes, whereas the reverse mutations are those from a mutant-type back to wild-type. In general, forward mutants may be broadly classified into visible, or morphological mutants, and biochemical mutants. In a sense, all mutants are induced by mutation of certain gene(s) which affects some biosynthetic pathways. Many biochemical mutants cannot be distinguished by naked-eye observation. The alteration

of the pigment in the spores is one important colour mutation in fungal genetics. A particularly valuable type of colour mutant is the ascospore-colour mutants of linear asci. They have been used most fully to study the mechanism of genetic exchange at meiosis in Sordaria (Olive, 1956; Kitani and Olive, 1967 and 1969). The commonest biochemical mutants are auxotrophs which require the supplement of a specific nutrient in the medium used for the growth of wild-type strain. Spontaneous mutants can occur in all organisms but at an extremely low frequency. For example, Tatum and coworkers (1950) reported for Neurospora that out of 3,000 cultures tested, only one auxotrophic mutant was obtained. de Serres and Kolmark (1958) also concluded that mutation frequency at specific loci was very low, e.g. 10^{-6} conidia for ade-requirement in N. crassa. Therefore, it is necessary to induce artificially and select the mutants by suitable methods . The principal mutagenic agents are those which can modify the DNA structure including physical and chemical mutagens. Since the past decade, the mechanism of mutagens operation has been greatly understood and this fundamental research has led to their more rational use. Several mutation-inducing agents have been employed with fungi, e.g. UV-irradiation (ca 254 nm), X-irradiation, mustard gas, diethyl sulphate, EMS, nitrous acid, NTG, etc. Among them UV-irradiation

was commonly used in Aspergillus, Coprinus, Neurospora, Phycomyces, and Ustilago with satisfactory results (Burnett, 1975). It is usually found that no two fungi necessarily respond in the same manner to the same mutagenic agent. For instance, NTG which was a most promising chemical mutagen for both Escherichia coli and Saccharomyces cerevisiae, was no more effective with Coprinus lagopus than UV-irradiation (Moore, 1969). On the other hand, no mating type factor mutants were induced in Schizophyllum commune by UV or X-ray but were readily obtained by EMS treatment (Raper et al., 1965). In many cases, UV-irradiation is still frequently a potent mutagenic agent for fungi such as Ustilago maydis (Perkins, 1949) Penicillium chrysogenum (Mac Donald, 1968), Agaricus bisporus (Raper et al., 1972), Schizophyllum commune and Saccharomyces cerevisiae (Shneyour et al., 1978). The mechanism of UV-induced mutation has been reviewed by Witkin (1969). Most UV-induced mutations are introduced into the DNA molecule. This treatment tends to produce dimerization of adjacent pyrimidine bases within a single DNA strand. The double-carbon bonds of thymine are disrupted and the two thymine bases could be connected to form a thymine dimer. In vitro studies indicated that thymine dimerization might be the primary mutagenic effect produced by UV. The thymine-thymine dimers are formed more readily than

cytosine-thymine or cytosine-cytosine dimers (Wacker et al., 1962; Setlow et al. , 1965). Such dimers would distort the DNA helix and interfere with proper replication. However, dose and mutation rate is most usually a non-linear relationship which could rise with increasing dose to an optimum and then fall down sharply (Burnett, 1975). The damage caused by UV can be reversed before genetic material is permanently affected by exposing cells to visible light containing wavelengths in the blue spectrum. This repairing process, known as photoreactivation, has now been observed in numerous organisms including bacteriophages, bacteria and fungi (Setlow and Setlow, 1963; Setlow et al., 1965). Recovery from UV damage can also occur in the dark by the dark repair mechanisms which involve enzyme action (Boyce and Howard-Flanders, 1964; Setlow and Carrier, 1964).

Success in the induction of auxotrophic mutants following mutagenic treatment is usually low. A dose of UV at 99% killing of the cells in suspension would yield about one mutant per 100 survivors and lower doses of UV would give more survivors but fewer of these cells would be mutants (Woods, 1973). Mutant recovery could be improved by applying conditions which would select against non-mutant survivors or prototrophs. The earliest and most successful procedure was the penicillin selection technique which has been widely used in the isolation of

bacterial mutants. This technique was based on the inability of auxotrophic mutants to grow and divide on minimal medium but the other survivors which could grow would be continually killed by penicillin (Gorini and Kaufman, 1960). Application of this technique could enhance the proportion of mutants to 10% or 20% of the plated cells. This was followed by the routine use of enrichment techniques for the easy isolation of auxotrophs after mutagenic treatment in the studies with single-cell microorganisms. The penicillin technique has been successfully applied to algae (Shneyour and Avron, 1975; Toby and Kamp, 1975). Other antibiotics were also used as selection agents for enchancing the mutation rate such as nystatin which was used in Rhodotorula mucilaginosa (Cook, 1974), Penicillium chrysogenum (Mac Donald, 1968) and Aspergillus (Stanley and English, 1965); netropsin used in Saccharomyces cerevisiae (Young et al., 1976); and cycloheximide used in Schizophyllum commune and Saccharomyces cerevisiae (Shneyour et al., 1978).

Auxotrophs can be identified on a nutrient supplemented minimal medium when they would show growth on the minimal medium but not on the complete medium. Therefore, the procedures employed for identifying auxotrophs are essentially a series of successive tests in which the possible requirements of nutrients are pro-

gressively deduced. The classical method is to inoculate auxotrophs on minimal medium plus (a) a vitamin mixture, (b) a mixture of amino acids, and (c) purines and pyrimidines mixtures from nucleic acid hydrolysate. When growth can be achieved on one of these supplemented media, the mutant is tested on a series of minimal media to which each component of the mixture is added individually. A considerably improved method has been suggested and used by Holliday (1956). This method involves a series of media each containing a number of substances and each substance occurs in only two media containing different components. If a certain mutant is unable to grow on any of the test media, then the corresponding colony would be absent.

B. RESULTS

I. UV-killing Curves

Pleurotus spores were sensitive to UV with 98% killing after 2 min irradiation (Fig. 4.1). When compared to spores, *Pleurotus* mycelia were much more sensitive to UV and 30 sec irradiation resulted in 95% mortality (Fig. 4.2). Two chemicals were tested for their effect on *Pleurotus* mycelia. It was found that 10 µg per ml of cycloheximide could cause only 10% survival as compared to the control (Fig. 4.3). On the other hand, chlorte-

Fig. 4.1 Killing curve showing the effect of UV light on the survival of basidiospores of Pleurotus sajor-caju (P127).

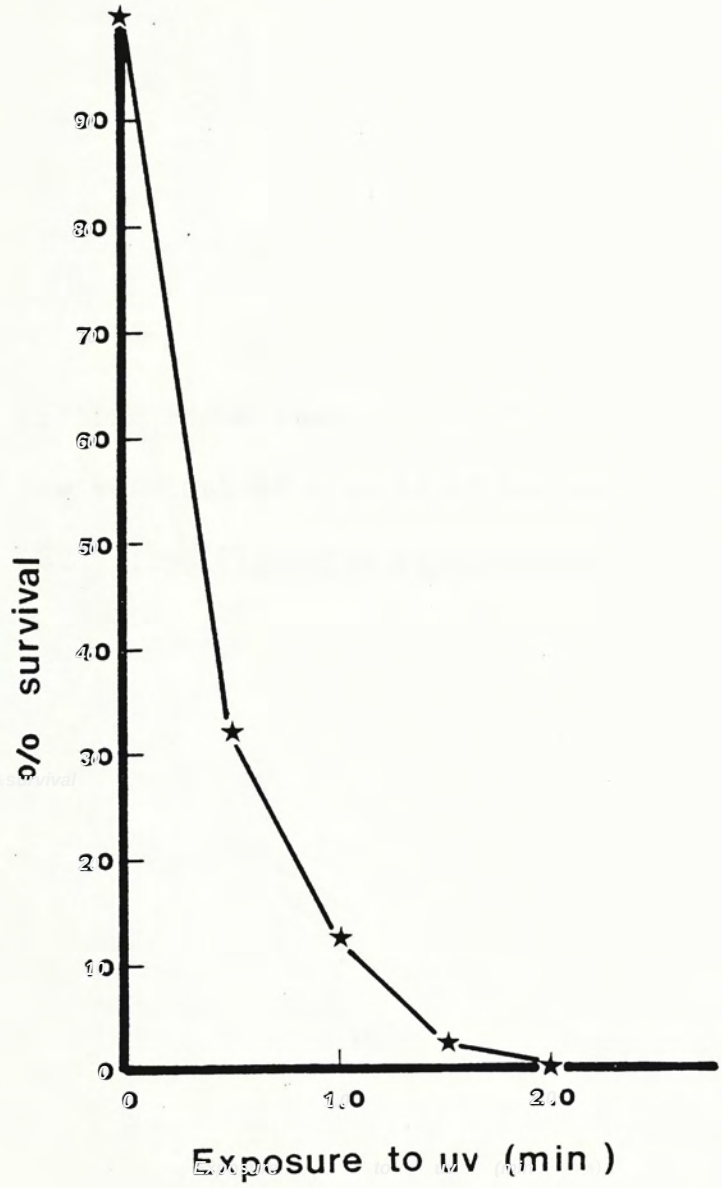

90
80
70
60
50
40
30
20
10
0
% survival
0
1.0
2.0
Exposure to uv (min)

Fig. 4.2 Killing curve showing the effect of UV light on the survival of mycelia of monokaryotic strain (S23) from Pleurotus sajor-caju.

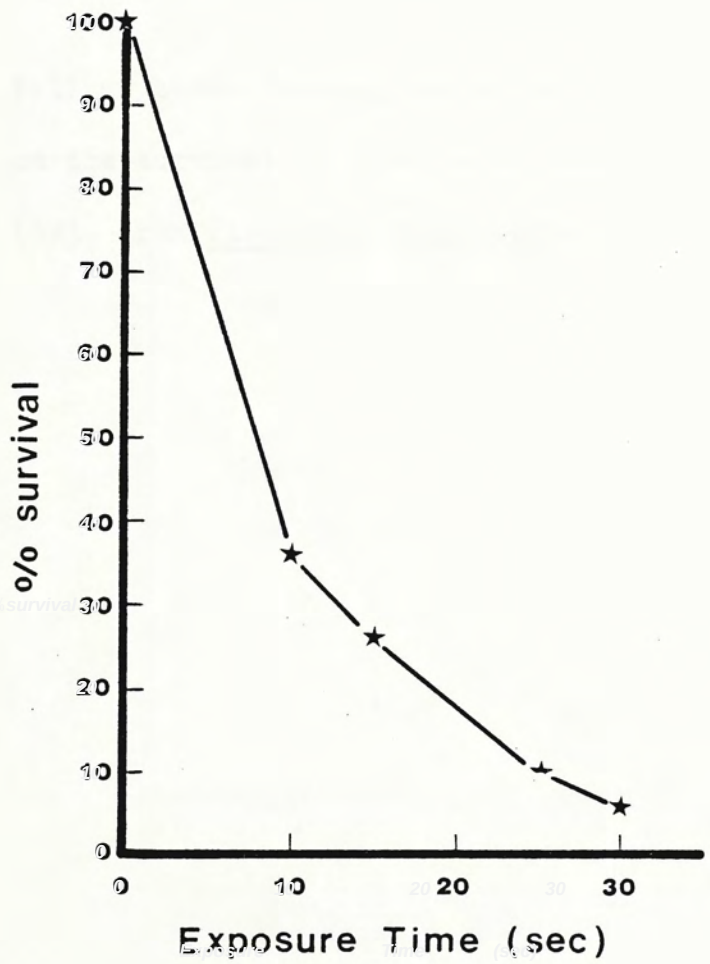
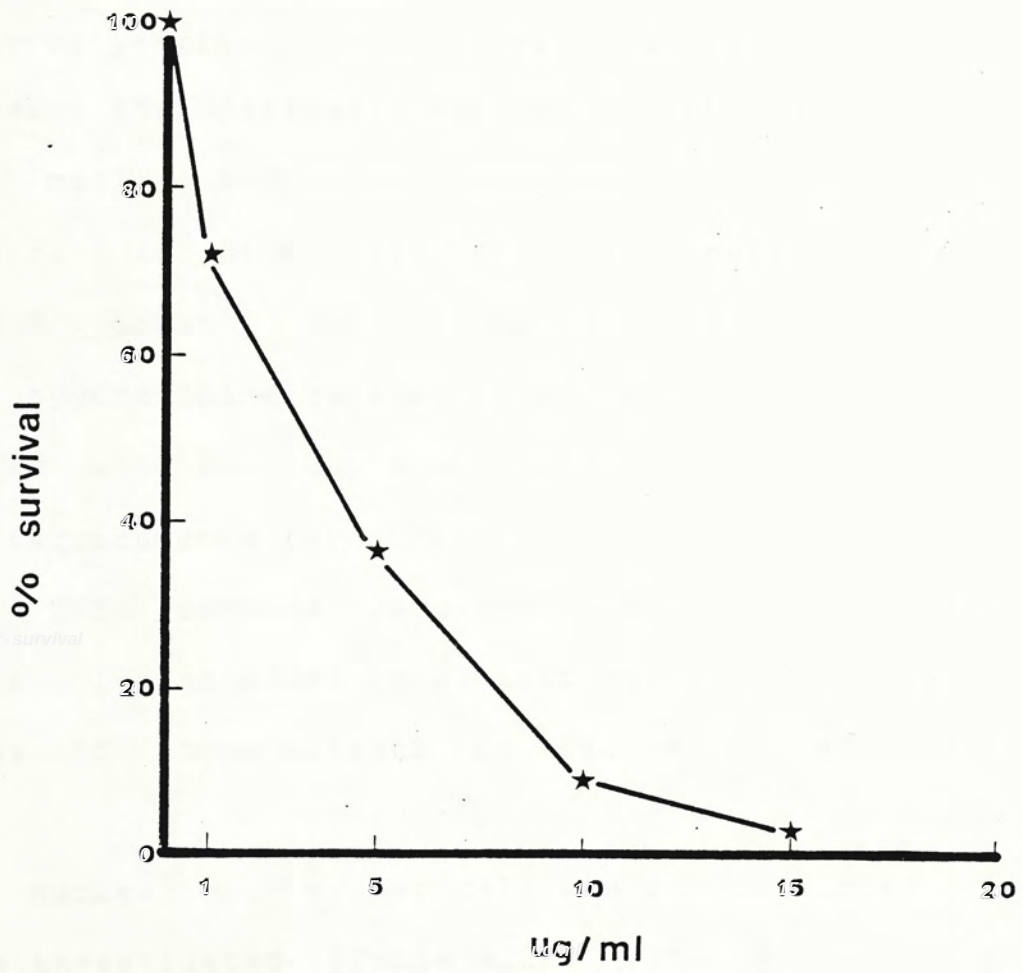

100
90
80
70
60
50
40
30
20
10
0
% survival
0
10
20
30
Exposure Time (sec)

Fig. 4.3 Killing curve showing the effect of cycloheximide on the survival of mycelia of monokaryotic strain (S23) from Pleurotus sajor-caju.


100
80
60
40
20
0
% survival
1
5
10
15
20
ug/ml

tracyclin had lethal effect of 50% on Pleurotus mycelia even at high concentration of 500 µg per ml (Fig 4.4).

II. Mutant Identification

After 7 - 8 day incubation, survivors among the UV-treated spores germinated and formed colonies on complete medium. Using the Holliday's method, mutant Su42 was able to grow on minimal medium supplemented with Holliday's mixtures nos. 2 and 8, so this mutant apparently required folic acid for growth. Su72 appeared to require either adenine or hypoxanthine because it showed growth on medium supplemented with Holliday's mixtures nos. 1, 7, and 9. Nutrient requirements for other auxotrophs are showed in Table 4.1. This results were confirmed by the specific nutrient supplement added in minimal medium. The mating type class of these mutants was also determined (Table 4.1).

The nuclear number per cell was one in almost all auxotrophs investigated (Table 4.2). The formation of some binucleate cells could be the outcome of nuclear division. Dikaryotized mycelia, formed from pairing of Su42 x S22, Su223 x S23, Fu22 x F6, and Ful56 x F6 could further develop fruit bodies in tube cultures containing complete medium.

Attempts at auxotroph isolation by UV-irradiation of

Fig. 4.4 Killing curve showing the effect of chlortetracycline on the survival of mycelia of monokaryotic strain (S23) from Pleurotus sajor-caju.

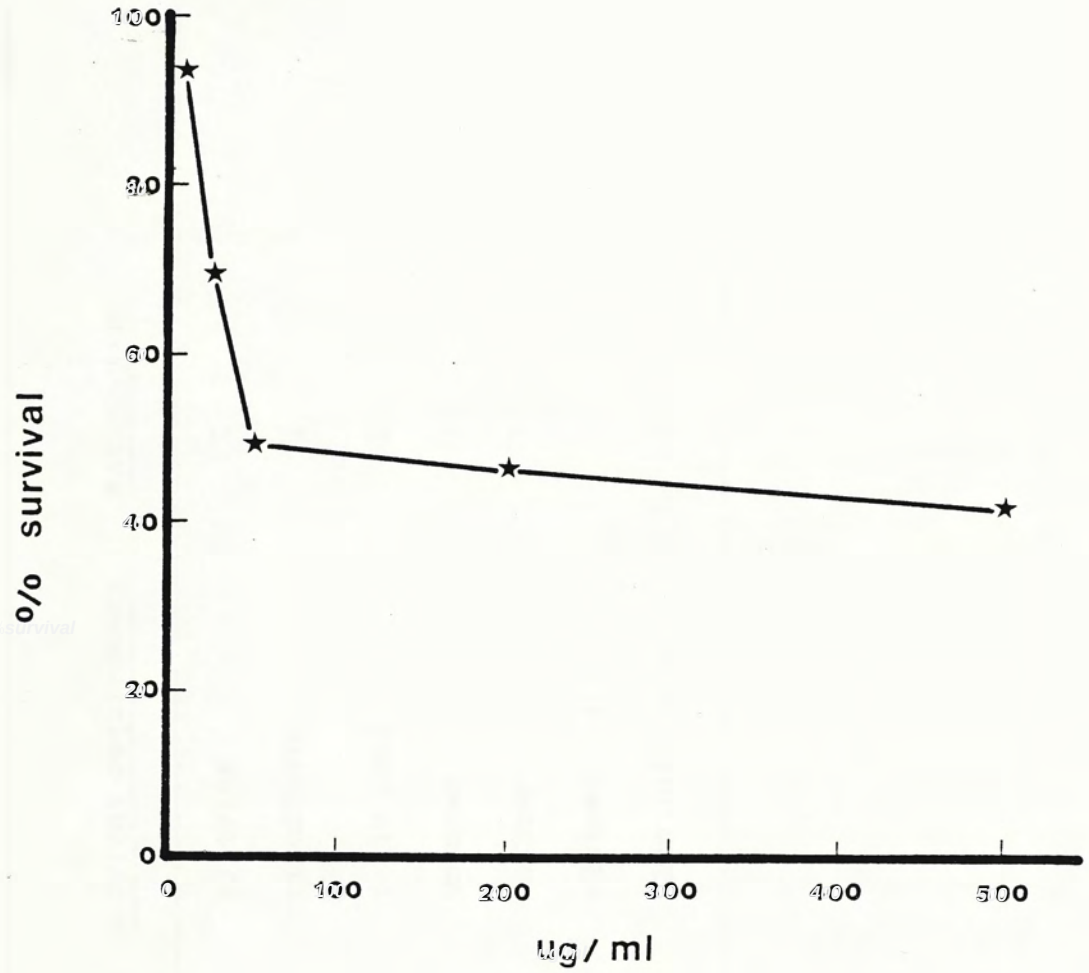

100
80
60
40
20
0
% survival
0
100
200
300
400
500
ug / ml

Table 4.1 Nutrient requirement and mating type of Pleurotus sajor-caju (Su) and P. florida (Fu) auxotrophs.

Auxotroph strain	Nutrient requirement	Mating type
Fu22	cytosine	I
Fu156	riboflavin	I
Su42	folic acid	IV
Su70	unknown	II
Su72	unknown	I
Su223	glutamic acid	I
Su309	nicotinic acid	II

Table 4.2 Number of nuclei in apical cells and subapical cells of mycelia of some auxotrophs.

Auxotroph	Apical cell		Subapical cell	
	1	2	1	2
Fu22	20	3	29	0
Fu156	27	0	33	0
Su42	13	1	41	2
Su223	26	0	38	0
Su309	17	2	25	1

mycelia were not successful.

III. Mating Reactions between Mutants of P. sajor-caju and of P. florida.

For each of the matings, Su42 x Fu22, Su42 x Ful56, Su223 x Fu22 and Su223 x Ful56, the two strains were inoculated on complete medium and allowed to grow for 10 days. The contact zone did exist between all pairing strains (Fig. 4.5). Small mycelial plugs, cut from the contact zone of the mated mycelial colonies, were subcultured on fresh minimal medium for 5 days, but no growth occurred .

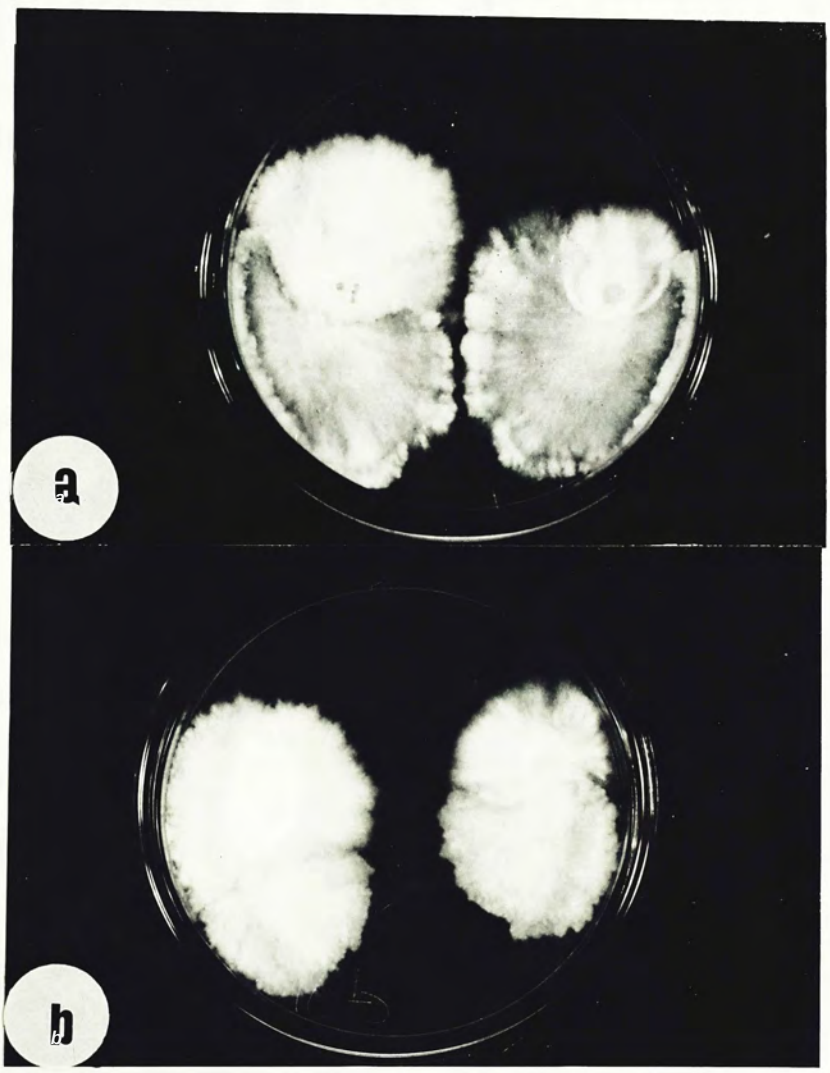
C. DISCUSSION AND CONCLUSION

Above results showed that UV was also a potent mutagenic agent for P. sajor-caju and P. florida. It is found the mycelia were more susceptible to UV-irradiation than the spores. It was not surprising, as the cell wall of spores was usually thicker and more complicated than that of mycelia, and this could make it more difficult for UV to penetrate into the spore nuclei. As mentioned by Burnett (1975), UV-irradiation has been successfully employed as a useful mutagen for fungi with hyaline

Fig. 4.5 Resulting colonies showing mating reactions among the pairings of monokaryotic mutants from P. sajor-caju (Su42 and Su223) and monokaryotic mutants from P. florida (Fu22 and Fu156).

(a) Su42 x Fu22 (left), and Su42 x Fu156 (right).

(b) Su223 x Fu22 (left), and Su223 x Fu156 (right).


a
b

spores but not with heavily pigmented or thick-walled spores because of absorption losses. For example, the spores of Volvariella volvacea which were pink in colour, were more resistent to UV and required 7 min with a higher dose (39 ergs per sec per cm^2) for 90% mortality (Santiago, 1982a). Prototrophic spores could germinate well and develop small colonies in liquid minimal medium after 1 - 2 day culture. Therefore, the germinated prototrophic spores could be easily retained by the glass wool through filtration and only the slow-growth prototrophic spores and auxotrophic spores remained in the liquid minimal medium. This technique allowed the enhancement of the recovery frequency of auxotrophs from the UV-treated spore suspension without any toxic effect on the survivors. All the auxotrophic mutants of Pleurotus were obtained by this method. The unsuccessful use of cycloheximide for the enrichment of auxotrophs could be ascribed to many factors such as the suitable concentration and the optimum period of treatment. Cycloheximide could result in a 500-fold for yeasts and a 140-fold for Schizophyllum commune than those without treatment when NTG was used as mutagen for auxotrophic mutation (Shneyour et al., 1978). No effort was make to find out whether cycloheximide was suitable for use in Pleurotus.

The mating reactions between auxotrophic mutants of P. sajor-caju and P. florida were similar to those

described in Chapter 3. Moreoever, subcultures of inocula taken from the contact zone did not show mycelial growth. This result indicated that there was no association of the two complementary genomes by hyphal fusion.

CHAPTER 5

PROTOPLAST ISOLATION AND REVERSION

A. INTRODUCTION

I. Definition of a Protoplast

One of the most significant developments in the field of plant biology during recent years has been the isolation, culture and fusion of protoplasts (Cocking, 1972). In the strict sense, a protoplast is defined as the structure derived from a plant cell by the removal of the entire cell wall (Villanueva and Garcia-Acha, 1971; Peberdy, 1976). However, when the residue of the cell wall remains, or when it is not known whether or not such residue still remains on the cytoplasmic membrane, the term "spheroplast" is used. In such a case, the osmotic sensitivity alone appears to be an insufficient criterion because lysis or bursting of the cytoplasmic membrane also occurs even after the partial removal of the cell wall. In the case of filamentous fungi, protoplasts usually arise by just emerging through the pores leaving behind the empty cell wall after protoplast release. Again, it is also possible that some undigested cell wall would

still remain attached to the membrane of the protoplast. Therefore, it is difficult to define a protoplast accurately in fungal protoplast isolation. In practice, criteria used for defining protoplasts are osmotic fragility resulting in a spherical structure, and the observation on the liberation of the protoplast through a pore leaving behind the empty cell wall (Villanueva and Garcia-Acha, 1971).

II. Practical Applications of Isolated Protoplasts

The first enzymatic isolation technique was described for yeasts by Eddy and Williamson (1957) and for higher plants by Cocking (1960). Since then, many workers have been devising different methods for isolation and culture of protoplasts from other various plant groups including blue-green algae (Crespi et al., 1962), mosses (Binding, 1966), liverworts (Schieder, 1975), filamentous fungi (Bachmann and Bonner, 1959) and bacteria (Marquis and Corner, 1976).

Isolated protoplasts offer a means of tackling various fundamental research problems in the plant science. Because plant protoplasts are devoid of cell walls, they can be treated experimentally as cultured mammalian cells. The various uses of protoplasts in recent experiments in higher plants include: re-biosynthesis

of cell wall (Nagata and Takebe, 1970; Meyer and Abel, 1975; Burgess and Linstead, 1976); isolation of organelles such as chloroplasts (Wagner and Siegelman, 1975) and nuclei (Blaschek et al., 1974); high-efficient experimental virus infection (Takebe, 1975); and several genetic studies (Carlson, 1973). In fungi, protoplasts have been used for the investigation on the biosynthesis in Saccharomyces cerevisiae (Morgan, 1983) and the cell wall regeneration in Schizophyllum commune (de Vries and Wessels, 1975). The special value of protoplasts for the study of protein secretion from protoplasts and of the cell wall resynthesis has also been demonstrated in yeasts (Sentandreu et al., 1983). More recently, the protoplast technology has been becoming especially important because of their increasing implication in the studies of plant improvement by somatic hybridization (Peberdy, 1980). Though this fascinating field of research is still in its initial stage, it has already been playing an important role in opening up a new area in biological technology. For studying such an exciting new field, the success is clearly very dependent on the basic work on protoplast isolation and culture. In general, a successful protoplast isolation is mainly influenced by three major factors, namely, the lytic enzyme used for the cell wall digestion, the osmotic stabilizer for maintaining the protoplast intact, and the organism

itself (Peberdy, 1979).

III. Protoplast Isolation

There are three major groups of methods for the isolation of protoplasts: the mechanical method, the methods which involve the use of inhibitors and enzymatic methods. The mechanical or autolytic method is the oldest one which involves the plasmolysis of suitable tissues in high osmotic solution and deplasmolysis in low osmotic solution. One of the disadvantages of this method is that, during deplasmolysis, only a small number of cells which had their walls disrupted enough to allow the surviving protoplasts to emerge along the broken surface. In general, such a mechanical method cannot yield large number of protoplasts and so it is rather unreliable. The specific inhibition of cell wall synthesis, without affecting the cytoplasmic biosynthesis, has been used for protoplast preparation from gram-negative bacteria under conditions of active growth and in the presence of suitable osmotic stabilizers (Martin, 1983). However, such attempts with fungi have been much less successful (Villanueva and Garcia-Acha, 1971). Since workers have favoured the use of lytic enzymes for protoplast isolation, which is most efficient, this technique became well established (Peberdy, 1979). The en-

zymatic method which involves the use of enzyme complexes obtained from several microorganisms (Villanueva and Garcia-Acha, 1971; Peberdy, 1976). The major drawback of such enzyme complex preparation is time consuming and can be expensive. Several commercial enzymes are now available, such as glucanase, helicase, and sulphatase for different yeast species (Deutch and Parry, 1974; Foury and Groffeau, 1973; Shahin, 1972).

After Helix digestive juice was first reported to be usable as a lytic enzyme for the protoplast isolation in yeasts (Eddy and Williamson, 1959), it has been employed extensively in several different yeasts (Rodriguez and Villanueva 1962; Shahin, 1972; Foury and Groffeau, 1973; Deutch and Parry, 1974). The snail digestive juice was proved to be effective against some filamentous fungi, e.g., Neurospora crassa (Bachmann and Bonner, 1959; Emerson and Emerson, 1958), Aspergillus nidulans (Ferenczy et al., 1974), Cephalosporium acremorium (Fawcett et al., 1973), and Geotrichum candidum(Ruiz-Herrera and Bartnicki-Garcia, 1976). Later, several sources of snail enzymes have become commercially available, and thus led to the sound establishment of protoplast technology in such fungi. However, when snail juice was used alone, far less quantitative conversion of protoplasts in filamentous fungi has been achieved than in yeasts. This could be explained by the fact that cell wall diversity, both in

composition and architecture, is great in fungal species (Gander, 1974). The enzyme extracts from microorganisms with mycolytic have been demonstrated to be the most effective lytic enzyme mixtures for protoplast isolation (Villanueva and Garcia- Acha, 1971). From the study on the enzymes of Trichoderma harzianum with respect to protoplast isolation from Schizophyllum commune (de Vries and Wessels, 1973), it was found that S-glucanase, R-glucanase and chitinase were necessary for protoplast isolation from S. commune. On the other hand, cellulase and exo-laminarinase were the two important components in the lytic enzyme complex from Streptomyces satsumaensis which was suitable for protoplast isolation in Pythium (Sietsma and de Boer, 1973). Moreover, several experimental results revealed that enrichment of lytic enzyme complexes with the addition of specific polysaccharase constituents can enhance the protoplast yield or the speed of protoplast release. The addition of glucanases to snail digestive juice could increase both parameters in protoplast isolation from Schizosaccharomyces pombe (Houssett et al., 1975). Better protoplast yields were obtained from Penicillium chrysogenum when a preparation from Streptomyces gramineofasciens and a commercial cellulase were added to the Trichoderma enzyme extract (Anne et al., 1974). Speed of protoplast release could be enhanced when glucanase

was added to the lytic complex from T. harizanum (Morris, 1978). However, in many cases, it was found that only one of the commercial sources of lytic enzymes was not effective for protoplast isolation in filamentous fungi. The combination of commerical enzymes, helicase and cellulase, were used in Pythium (Sietsma and de Boer, 1973). Enzyme solution contained commercial chitinase and chitosanase which was prepared from Streptomycin was effective for Phycomyces protoplast isolation (Binding and Weber, 1974). More recently, Novozym 234 , which is a commercial product, has been reported to be highly effective for many fungal species (Hamlyn et al., 1981).

Due to the lack of external protection, protoplast will immediately lyse or burst during the process of release from the hyphal wall. Therefore, a stabilizer is essential to provide osmotic pressure for maintaining the protoplasts intact. A wide range of inorganic salts, sugar, and sugar alcohols have been used as osmotic stabilizers (Table 5.1).

The nature and concentration of a stabilizer could be an important factor in protoplast isolation and could affect the characteristic of protoplasts in many filamentous fungi. It was generally found that inorganic salts are more effective with filamentous fungi and sugar or sugar alcohols are more suitable for yeasts (Peberdy, 1979).

Both mycelia and spores can provide the

Table 5.1 Chemicals used as stabilizers for fungal protoplast isolation.

Stabilizer	Conc.(M)	Organism	Reference
KCl	0.6	Aspergillus nidulans	Peberdy & Isaac, 1976
	0.55	Penicillium chrysogenum	Anne et al., 1974
	-15.8atm	Schizophyllun commune	deVries & Wessels, 1972
$MgSO_4$	0.8	Candida utilis	Cascon & Villanueva, 1965
	0.8	Fusarium culmorum	ditto
	0.8	Geotrichum candidum	Decijewaard-Kloosterziel et al., 1972
	-15.8atm	Schizophyllum commune	deVries & Wessels, 1972
	0.6	Volvariella volvacea	Santiago, 1982
NaCl	0.55	P. chrysogenum	Anne et al., 1974
	0.70	A. chrysogenum	Hamlyn et al., 1981
$NaNO_3$	0.55	P. chrysogenum	Anne et al., 1974
NH_4Cl	0.55	P. chrysogenum	ditto
Mannitol	0.65	Pythium	Sietsma & deBoer, 1973
	1.0	Saccharomyces cerevisiae	Eddy & Williamson, 1959
	0.8	Candida utilis	Garcia Mendoza & Villanueva, 196
Rhamnose	0.55	Saccharomyces carlsbergensis	Eddy & Williamson, 1957
	0.55	Saccharomyces cerevisiae	ditto
Sorbitol	0.35	Phycomyces	Binding & Weber, 1974
	0.35-0.5	Mucor pusillus	Ohnuki et al., 1982
	0.35-0.5	M. miehei	ditto
	1.2	Saccharomyces cerevisiae	Hamlyn et al., 1981

materials for protoplast isolation in filamentous fungi. Protoplasts isolated from spores or conidiospores could be expected to be more homogeneous in their cytoplasmic components and in the number of nuclei. In general, the spore wall was found to be more resistant to lytic enzymes and there are only a few reports on the preparation of protoplasts from conidia (Chu and Alexander, 1972; Moore and Peberdy, 1976; Bos and Slakhorst, 1981). Although protoplasts isolated from mycelia vary in size and in the number of nuclei per protoplast, these vegetative cells are still the suitable and convenient material for filamentous fungi, especially for the monokaryotic mutant strains of Basidiomycetes which do not produce spores. The physiological state of the mycelia is also critical for protoplast release and mycelial age is important . Cells or mycelia from cultures at the exponential growth phase could give the best result for most species (Duell et al., 1964; de Vries and Wessels 1972; Peberdy et al., 1976).

IV. Protoplast Regeneration and Reversion

Protoplasts could regenerate in liquid medium as well as on solid medium when a suitable osmotic stabilizer is added to the nutrient medium. Different kinds of osmotic stabilizers have a significant effect on protoplast

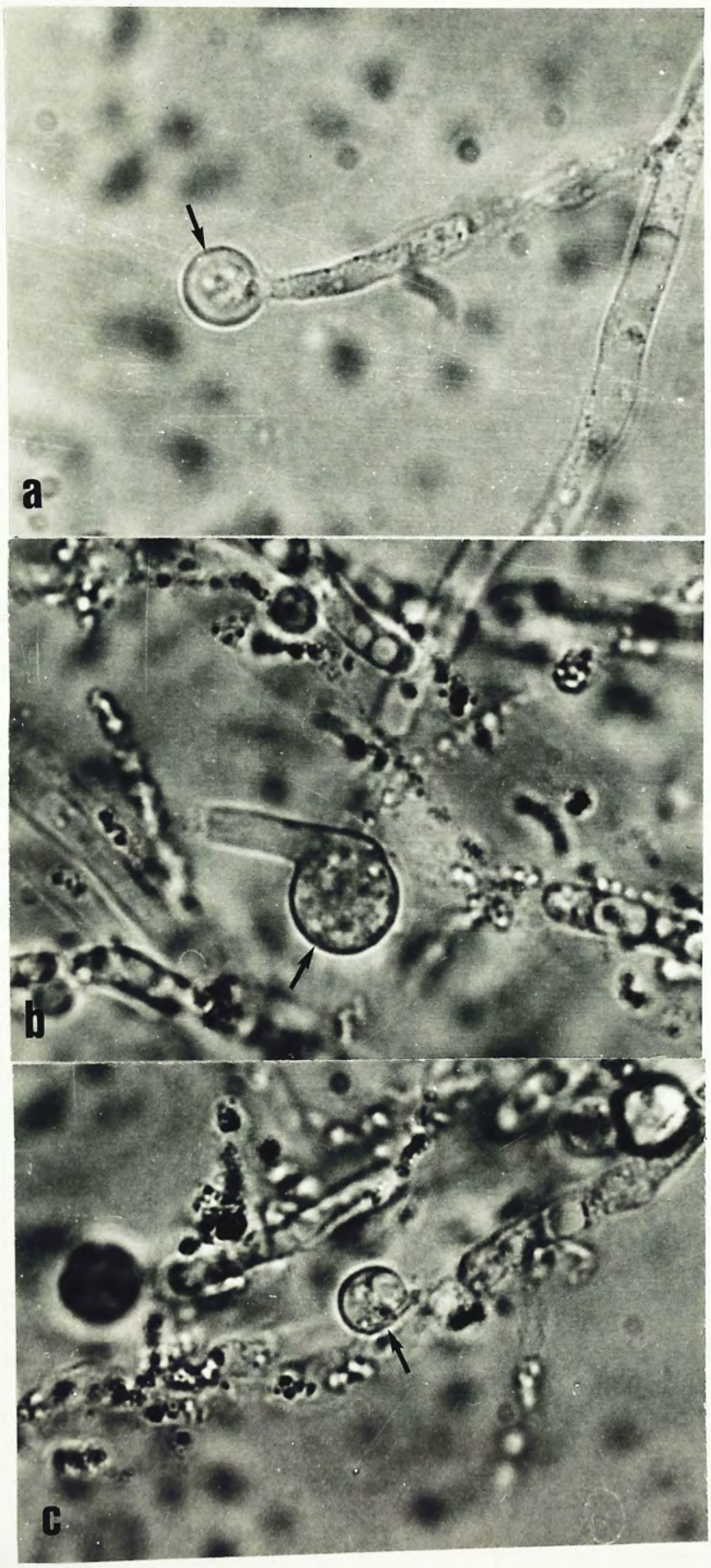
regeneration and reversion. In the case of Pythium, inorganic salts including NaCl, $MgSO_4$, KCl, and NH_4Cl could inhibit regeneration completely and so only organic compounds could be used (Sietsma and de Boer, 1973). On the other hand, inorganic salts were suitable for protoplast reversion of Schizophyllum commune (de Vries and Wessels, 1975) and Aspergillus sp. (Kevei and Peberdy, 1977). Carbon and nitrogen sources as components in the medium also had significant effect on protoplast reversion in V. volvacea (Santiago, 1982).

B. RESULTS

I. Conditions for Protoplast Isolation

For the preliminary study on the protoplast isolation in Pleurotus, the monokaryotic strains of P. sajor-caju (S23) and P. florida (F11) were used. After 30 min incubation in the osmotically stabilized lytic enzyme solution, some protoplasts were released from the hyphal tips or the immediate subapical regions (Fig. 5.1, a and b). Protoplasts were also released through the pores along the older parts of mycelia after a longer incubation time (Fig. 5.1, c). Using phase contrast microscopy, it was found that the cytoplasm in one mycelial cell was generally repeatedly constricted during extrusion

Fig. 5.1 Protoplasts (indicated by arrows) released from mycelium of Pleurotus sajor-caju (S23).
Magnification 1,000x, phase contrast.
(a) Protoplast released from hyphae tip.
(b) Protoplast released from hyphae tip.
(c) Protoplast released from the subapical region.


a
b
c

and divided into two or more spherical bodies.

The protoplast yield vs incubation period followed a sigmoidal pattern (Fig. 5.2). A maximum yield was obtained after 6 hr incubation and prolonged incubation up to 20 hr did not increase the protoplast yield. However, mycelial debris always remained in the lytic solution. There was no great difference in protoplast yield between these two different Pleurotus species.

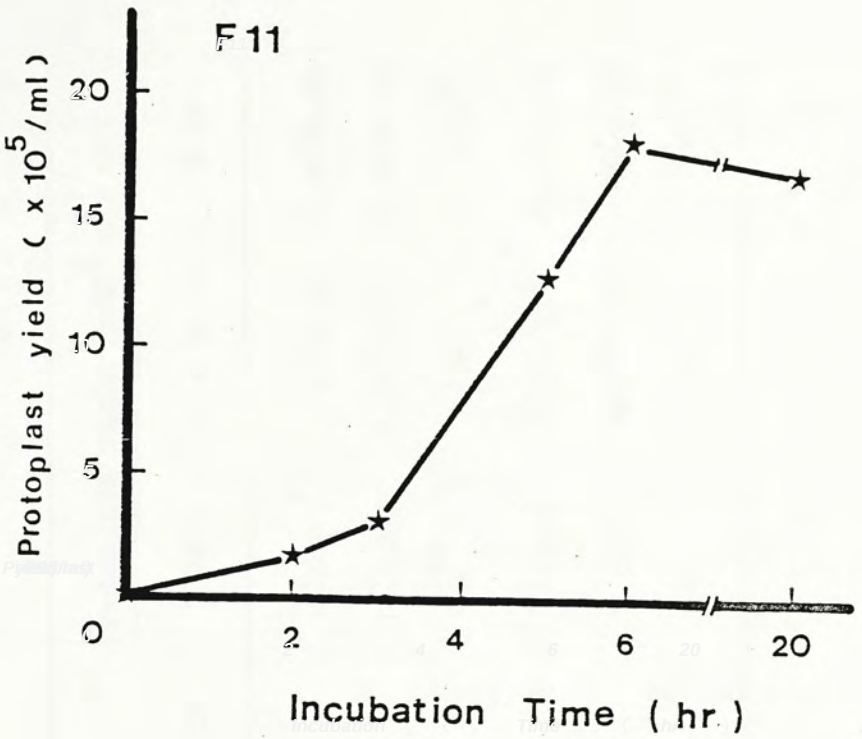
(a) Effect of lytic enzyme

From more detailed study, it was found that there was no great difference in protoplast yield when 10 mg per ml of Novozym 234 was used instead of the combination of Novozym 234 and Cellulase CP. However, when only 10 mg per ml of Cellulase CP was used, the protoplast yield was very low even after 6 hr incubation (Table 5.2). A combination of Novozym 234 and Cellulase CP at low concentration of 5 mg per ml appeared to give a satisafactory protoplast yield for Pleurotus.

(b) Effect of osmotic stabilizers and concentration

The protoplast yield was affected by the osmotic stabilizers used in the lytic enzyme solution. Both inorganic salts and organic compounds were used to compare their efficiency on protoplast isolation. Among the stabilizers tested, inorganic salts seemed to be better than the organic stabilizer and $MgSO_4$ appeared to be the

Fig. 5.2 Effect of incubation time on protoplast release from monokaryons from Pleurotus florida (F11), and from P. sajor-caju (S23). Mycelia (20mg per ml) were incubated at 28 °C with enzyme complex solution (Novozym 234 + Cellulase CP, 5mg per ml) containing 0.6 M $MgSO_4$ at pH 5.8.


F11
Protoplast yield (x 10^5/ml)
20
15
10
5
0
2
4
6
20
Incubation Time (hr)

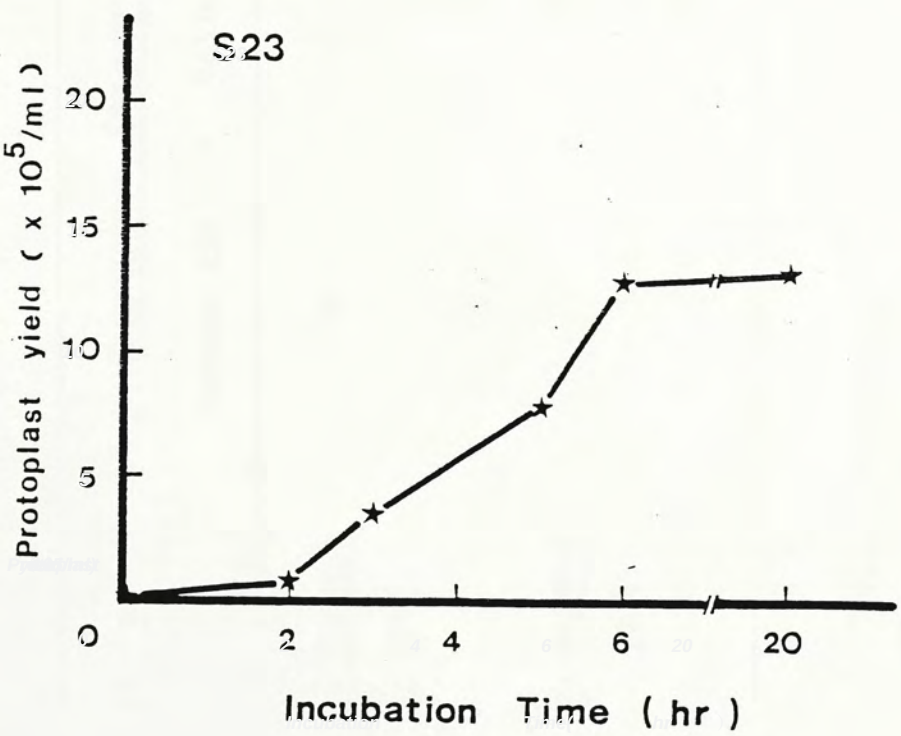

S23
Protoplast yield (x 10^5/ml)
20
15
10
5
0
2
4
6
20
Incubation Time (hr)

Table 5.2 Effect of concentration of Novozym 234 and Cellulase CP in the enzyme complex solution on protoplast release from Pleurotus.

Strain	Enzyme conc. (mg per ml)		Protoplast yield ($x10^6$ per ml)		
	Novozym 234 +	Cellulase CP	2 hr	4 hr	6 hr
P. florida (F11)	10	0	0.16 ± 0.01*	1.31 ± 0.14	1.96 ± 0.23
	5	5	0.18 ± 0.02	1.29 ± 0.13	1.80 ± 0.22
	0	10	-**	-	$1x10^4$
P. sajor-caju (S23)	10	0	0.18 ± 0.04	1.37 ± 0.18	1.63 ± 0.19
	5	5	0.17 ± 0.04	1.29 ± 0.15	1.30 ± 0.18
	0	10	-	-	$1x10^4$

*Standard deviation.

**Not counted

best for protoplast isolation (Table 5.3). $MgSO_4$ and KCl could give a 18% and a 16% increase in yield respectively compared to that of mannitol.

The protoplast yield was affected by the concentration of the stabilizer in the lytic solution. The optimum concentration of $MgSO_4$ was 0.6 M. The protoplasts observed in 0.2 M and 0.3M $MgSO_4$ could have just been released from the mycelia (Fig. 5.3).

Osmotic stabilizers also had different effect on the morphology of protoplasts when $MgSO_4$ was used as stabilizer. Many protoplasts had a large vacuole which occupied the most part of the protoplast (Fig. 5.4). Protoplasts released with KCl or mannitol as stabilizer contained several small vacuoles.

(c) Effect of pH

The optimum pH for protoplast isolation was determined using a 0.01 M phosphate buffer which contained 0.6 M $MgSO_4$. The stabilizer system was adjusted in the range of pH 4.5 to 6.5. It was surprisingly found that the highest protoplast yield was obtained at a low pH of 5.0 (Fig. 5.5).

(d) Effect of mycelial age

Pleurotus mycelia could grow well in the liquid medium. The mycelial growth rates at the linear phase after one day are shown in Fig. 5.6. However, protoplast release

Table 5.3 Effect of different osmotic stabilizers on protoplast release from *Pleurotus sajor-caju* (S23).

Stabilizer (0.6 M)	Protoplast yield ($x10^6$ per ml)		
	2 hr	4 hr	6 hr
$MgSO_4$	0.16 ± 0.04*	1.72 ± 0.20	2.34 ± 0.21
KCl	0.15 ± 0.06	1.62 ± 0.33	2.10 ± 0.17
Mannitol	0.14 ± 0.06	1.61 ± 0.32	1.98 ± 0.36

*Standard deviation.

Fig. 5.3 Effect of concentration of $MgSO_4$ on protoplast yield from Pleurotus sajor-caju (S23). Mycelia (20 mg per ml) were incubated for 6 hr at 28 C with enzyme complex solution containing Novozym 234 + Cellulase CP (5 mg per ml), pH 5.8.

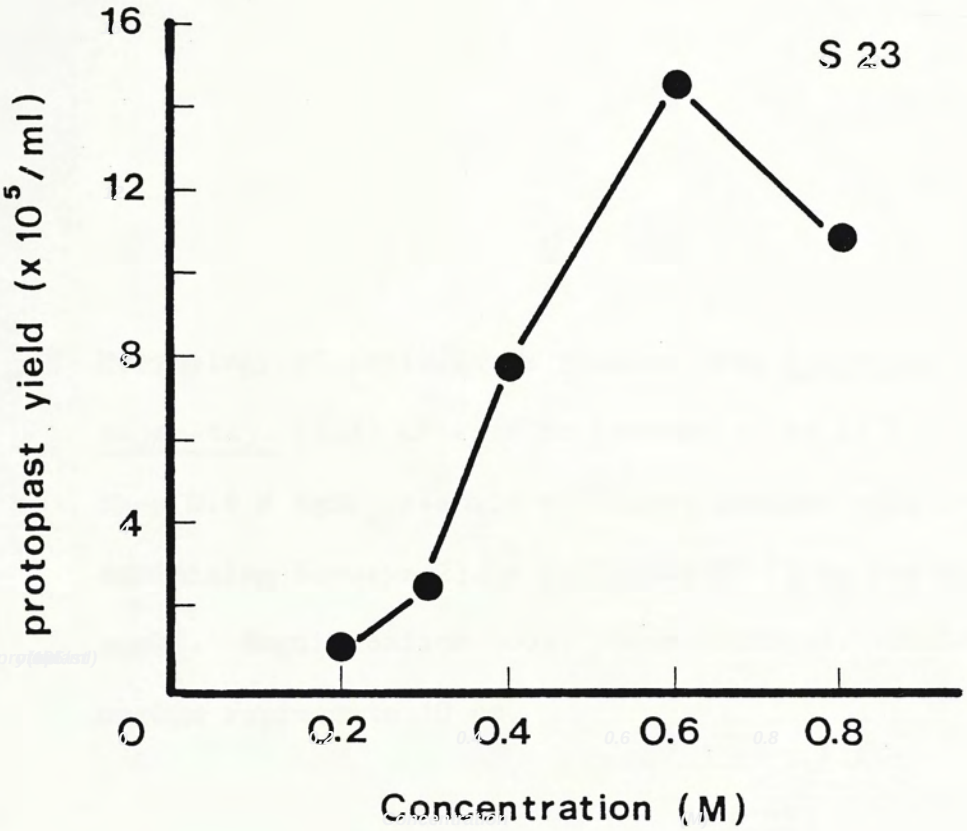

S 23
protoplast yield (x 10^5/ml)
16
12
8
4
0
0.2
0.4
0.6
0.8
Concentration (M)

Fig. 5.4 Morphology of protoplasts release from Pleurotus sajor-caju (S23) after 6 hr incubation at 28 °C in a 0.6 M $MgSO_4$ stabilized enzyme complex solution containing Novozym 234 + Cellulase CP (5 mg per ml each). Magnification 300x, phase contrast. Scale marker represents 10 um.

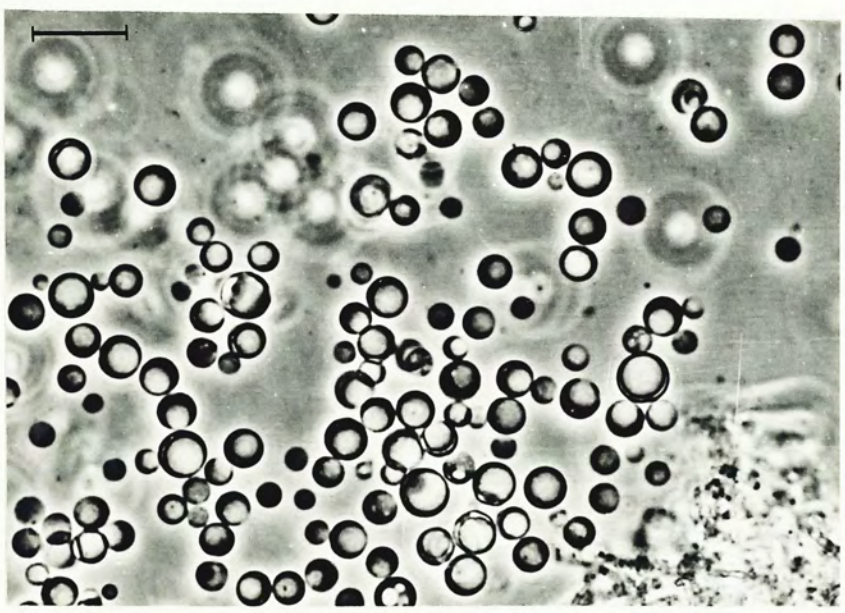


Fig. 5.5 Influence of pH on the protoplast yield from Pleurotus sajor-caju (S23). Experimental conditions were identical to those in Fig. 5.2.

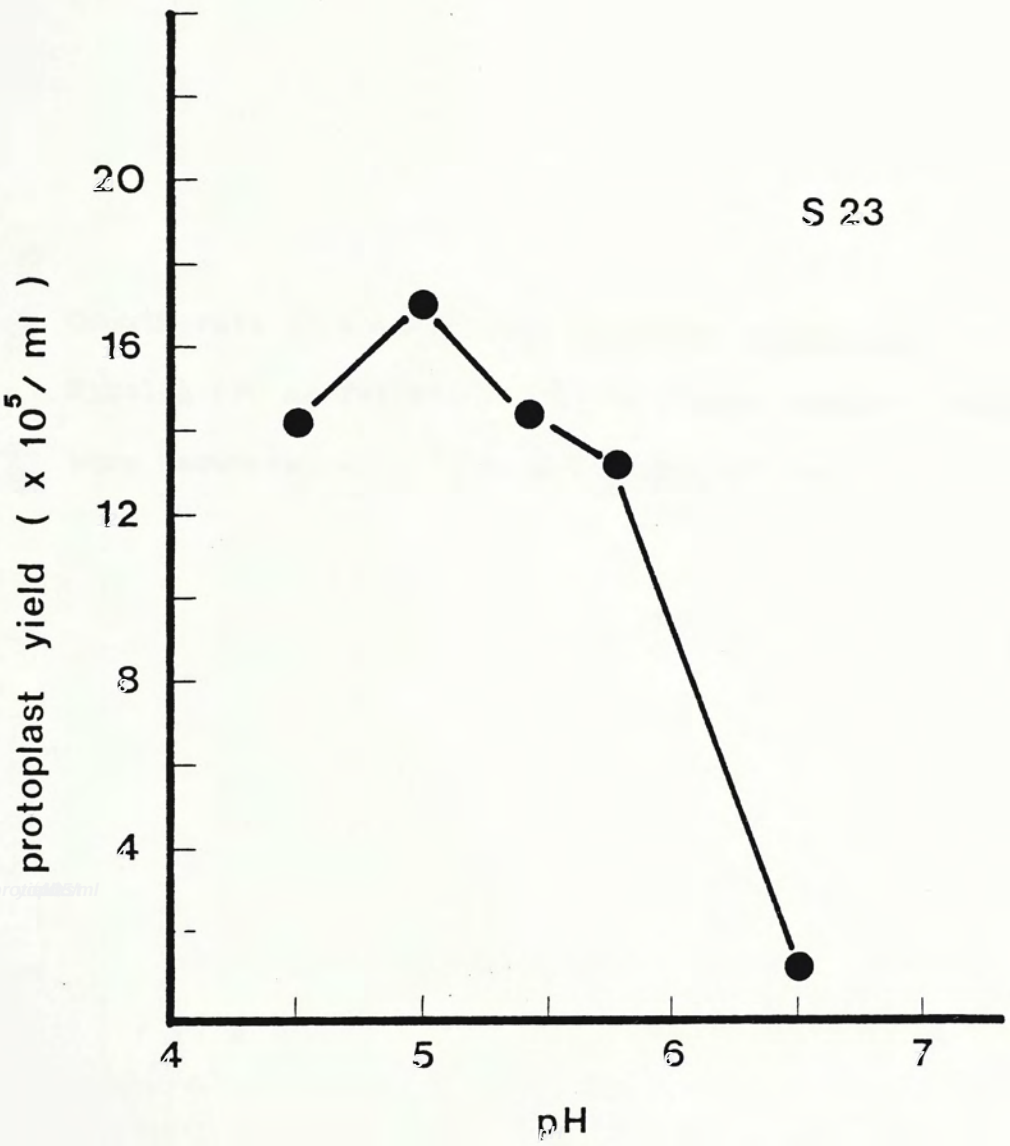
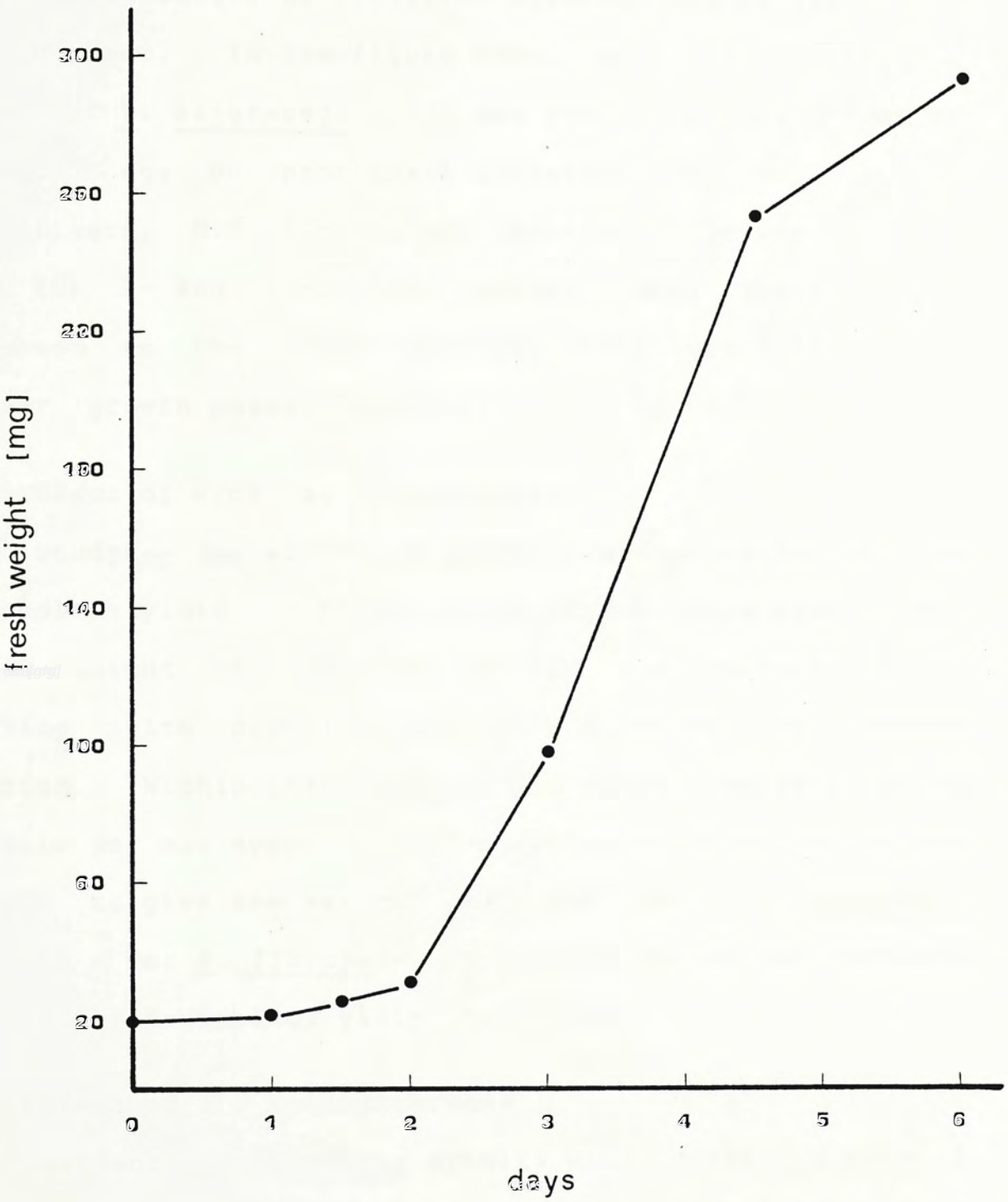

S 23
protoplast yield (x 10^5/ ml)
20
16
12
8
4
4
5
6
7
pH

Fig. 5.6 Growth rate of monokaryotic Pleurotus sajor-caju (S23). Mycelia (20 mg fwt per 20 ml) in liquid complete medium were incubated at 28°C on a shaker (120 rpm).


300
260
220
180
140
100
60
20
fresh weight [mg]
0
1
2
3
4
5
6
days

was very sensitive to different mycelial age at the linear growth phase. In the figure here, only one monokaryotic strain of P. sajor-caju (S23) was tested for the effect of mycelial age on protoplast isolation with two osmotic stabilizers, 0.6 M $MgSO_4$ and mannitol. It was obvious that the 1- and 2-day old cultures gave higher yield compared to the other culture which was still at the linear growth phase (Fig. 5.7).

(e) Effect of mycelial concentration

For studying the effect of mycelial concentration on the protoplast yield, 2 monokaryotic strains were used. The fresh weight of 2-day old mycelia was measured after washing with distilled water and dried by vacuum suction. Within the concentration range from 10 to 80 mg mycelia per ml, mycelial concentration of 20 mg per ml was enough to give the maximum yield for the P. sajor-caju strain. For P. florida, 40 mg mycelia per ml was required for better protoplast yield (Fig. 5.8).

(f) Effect of 2 - mercaptoethanol

Pretreatment of Pleurotus mycelia with 2-mercaptoethanol prior to digestion with lytic enzymes could stimulate the protoplast release. With the concentration of 200 mM, 2 - mercaptoethanol enhanced protoplast yield 10 - fold. Moreover, the pretreatment of 0.6 M $MgSO_4$ could also stimulate the 5 times protoplast yield (Fig. 5.9).

Fig. 5.7 Effect of mycelial age on protoplast yield from Pleurotus sajor-caju (S23). Mycelia (20 mg per ml) were incubated at 28°C with an enzyme complex solution (Novozym 234 + Cellulase CP, 5 mg per ml) containing $MgSO_4$ or mannitol as the osmotic stabilizer.

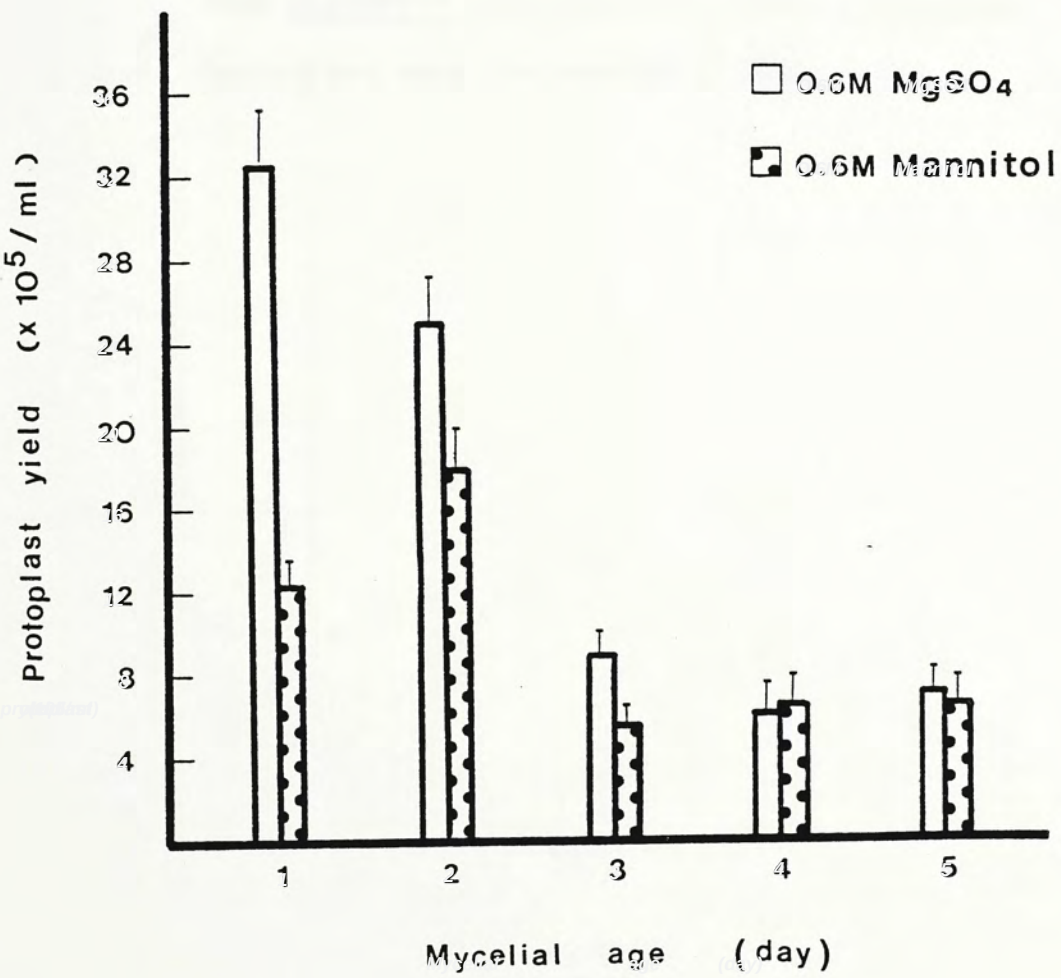

0.6M MgSO4
0.6M Mannitol
Protoplast yield (x 10^5 / ml)
36
32
28
24
20
16
12
8
4
1
2
3
4
5
Mycelial age (day)

Fig. 5.8 Effect of mycelial concentration on protoplast yield from Pleurotus florida (F11) and P. sajor-caju(S23). Conditions were the same as in Fig. 5.2.

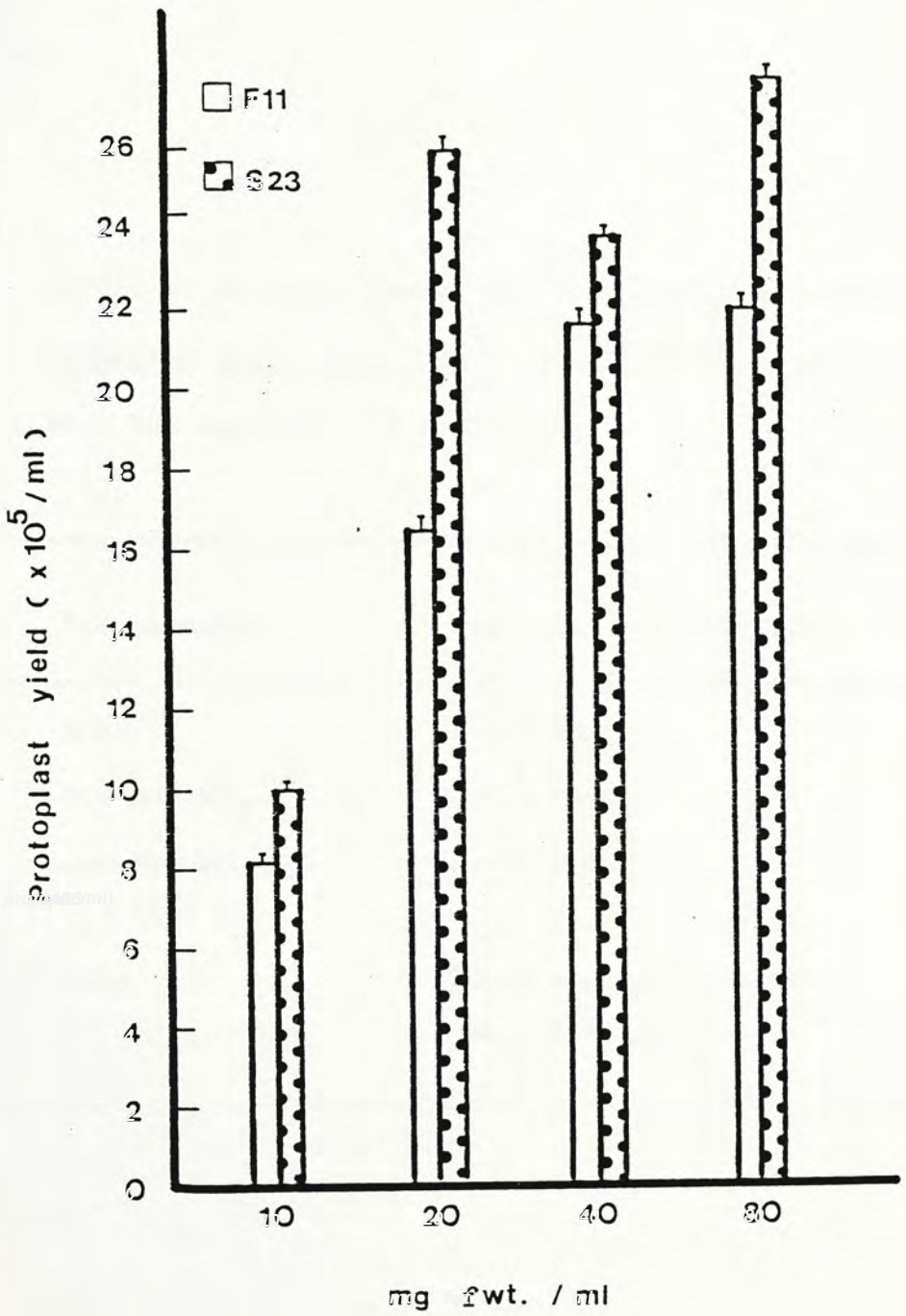
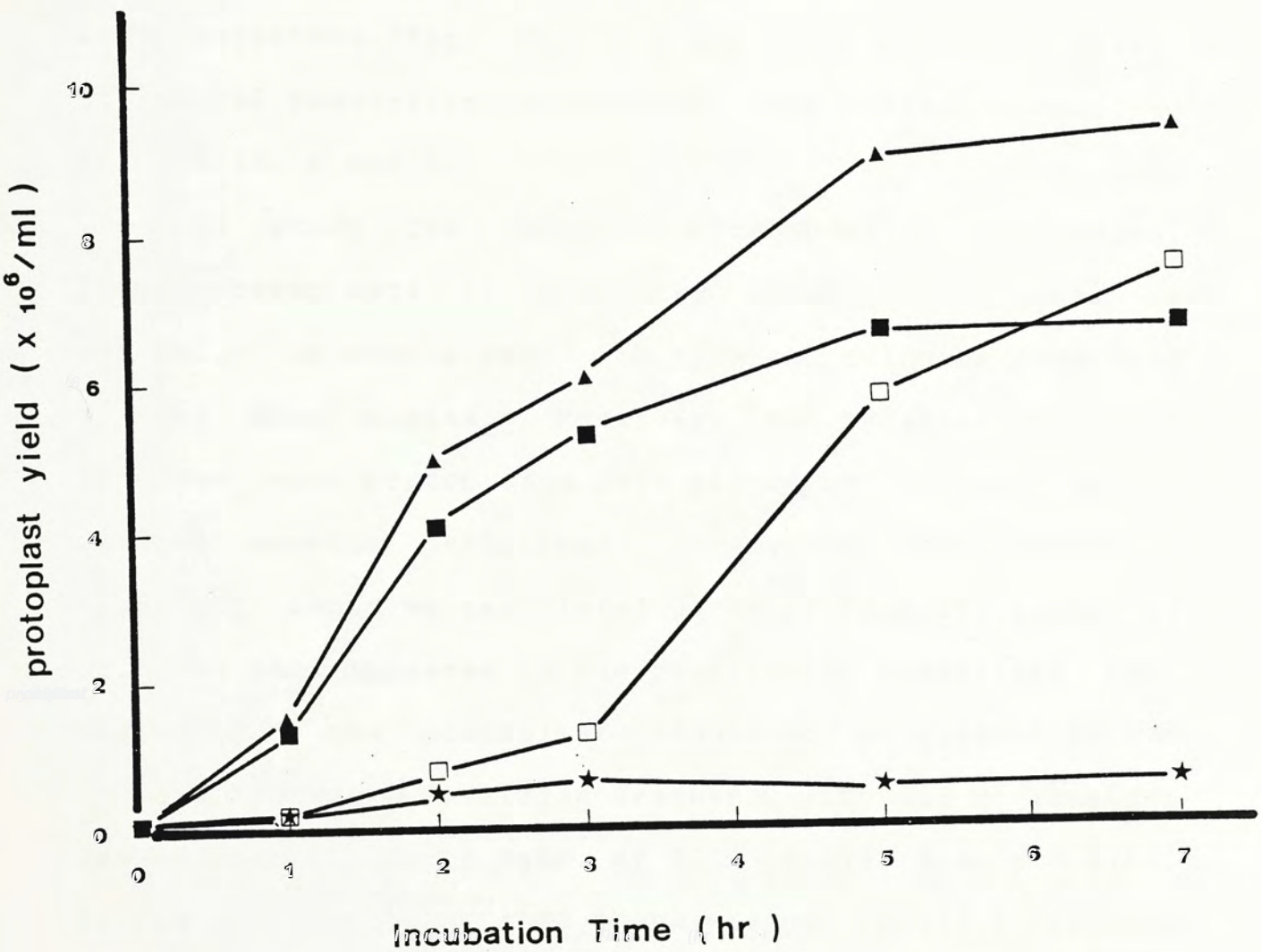

F11
S23
26
24
22
20
18
16
14
12
10
8
6
4
2
0
Protoplast yield (x 10^5 / ml)
10
20
40
80
mg fwt. / ml

Fig. 5.9 Effect of mercaptoethanol on protoplast yield from Pleurotus sajor-caju (S23). Experimental conditions were the same as in Fig. 5.2.

Symbol	Pretreatment	Stabilizer in Incubation
★	none	0.6 M $MgSO_4$
■	0.6 M $MgSO_4$	0.6 M $MgSO_4$
▲	mercaptoethanol (200 mM)	0.6 M $MgSO_4$
□	none	50 mM mercaptoethanol in 0.6 M $MgSO_4$


protoplast yield (x 10^6/ml)
0
2
4
6
8
10
0
1
2
3
4
5
6
7
Incubation Time (hr)

II. Regeneration and Reversion of Protoplasts

The development of hyphal tubes from protoplasts required 18-20 hr in the liquid complete medium supplemented with 0.6 M mannitol. Many of the regenerated protoplasts showed normal hyphae with one or more yeast-like structure (Fig. 5.10, c and d). In a few cases, chains of yeast-like structures were also observed (Fig. 5.10, a and b).

To study the reversion frequency on agar medium, lysed protoplasts in distilled water served as control. In such a case, no mycelial colonies developed on the agar plates. Moreover, no mycelial colonies appeared when protoplasts were plated on the agar medium without osmotic stabilizer. Therefore, the reversion frequency could be calculated directly from the number of colonies that appeared on the osmotically stabilized agar medium and the protoplast count of an aliquot plated on the medium. Reversion frequency with 0.6 M mannitol was higher than with $MgSO_4$ or KCl (Table 5.4 and 5.5). It was worth noticing that there was no great difference in reversion frequencies of protoplasts grown on the complete medium and on the minimal medium with different stabilizers. Nevertheless, the mycelia grew much better on a complete medium than on a minimal medium. However, the reversion frequency was very low and only ranged from 4 - 5%.

Fig 5.10 Patterns of morphological development of the protoplasts from Pleurotus sajor-caju strain (S23). Magnification 450x, phase contrast. Scale markers represent 10 µm.

(a) and (b) Chains of yeast-like structure developed from protoplasts.

(c) Regenerated protoplast with normal hypha.

(d) Regenerated protoplast with yeast-like structure (indicated by arrow) and normal hypha.

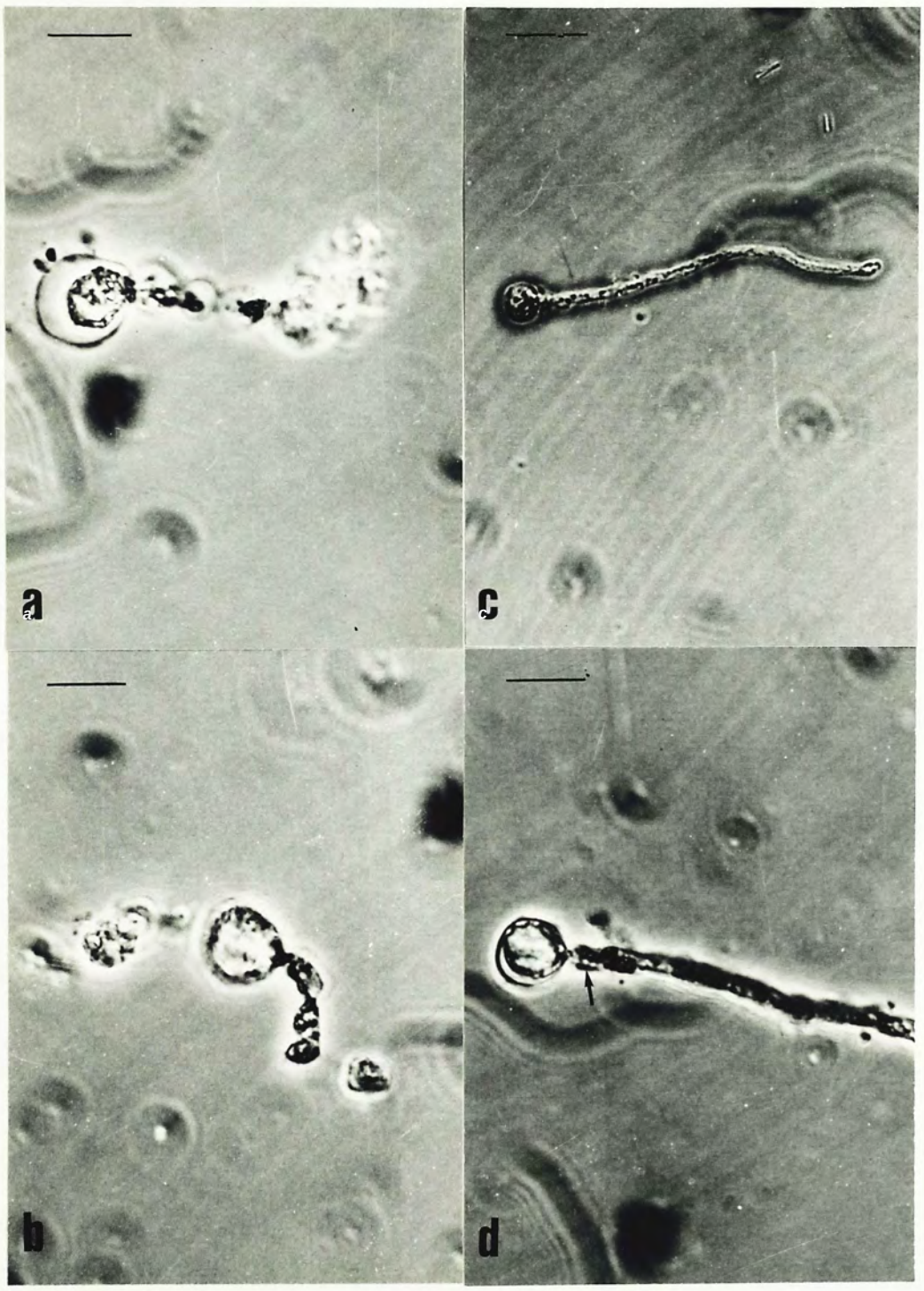

a
c
b
d

Table 5.4 Comparsion of the effect of different osmotic stabilizers, added to top agar, on protoplast reversion of the two Pleurotus strains.

Stabilizer (0.6 M)	Protoplast reversion (%)	
	P. florida (F11)	P. sajor-caju (S23)
$MgSO_4$	1.52 ± 0.21*	2.03 ± 0.54
KCl	1.10 ± 0.14	1.95 ± 0.25
Mannitol	2.32 ± 0.39	2.61 ± 0.34

*Standard deviation.

Table 5.5 Comparsion of the effect of complete medium and minimal medium on protoplast reversion of *Pleurotus sajor-caju* strain (S23).

Stabilizer in top agar (0.6 M)	Protoplast reversion (%)	
	Minimal medium	Complete medium
$MgSO_4$	3.10 ± 0.44*	4.03 ± 0.59
KCl	4.08 ± 0.24	3.98 ± 0.64
Mannitol	5.01 ± 0.69	5.13 ± 0.36

*Standard deviation.

C. DISCUSSION AND CONCLUSION

I. Conditions for Protoplast Isolation

(a) Effects of lytic enzyme

The results obtained demonstrated the success in the protoplast preparation for *Pleurotus* species. The protoplast yield in a lytic enzyme solution was influenced by several factors, but the most important one was the suitable enzyme(s) used.

Isolation of protoplasts from different fungal species requires specific lytic enzymes for digestion of their cell walls. However, only a few suitable enzymes have been available commercially and workers have often had to produce the lytic enzyme complex from microorganisms in their laboratories (Villanueva and Garcia-Acha, 1971; Peberdy, 1976). Among several commercially available enzymes, Novozym 234 has been proved as the most efficient enzyme for many filamentous fungi since it contained high levels of β-D-glucanase and chitinase activities (Hamlyn et al., 1981). Cellulase CP only contained 30% of chitinase activity when compared to Novozym 234. For *Pleurotus*, cellulase CP used alone was ineffective for protoplast isolation. Thus, the cell wall of *Pleurotus* protoplasts appeared to contain a great portion of chitin. However, the combination of Novozym

234 and cellulase CP at low concentration could give satisfactory protoplast yield as the high concentration of Novozym 234 did. Nevertheless, the protoplast yield was low when compared with Aspergillus, Penicillium, and Saccharomyces (Hamlyn et al., 1981). The maximum yield was obtained only after 6 hr incubation which was much longer than that for Schizophyllum commune (de Vries and Wessels, 1972), Aspergillus nidulans (Peberdy and Issac, 1976) and Volvariella volvacea (Santiago, 1982).

(b) Effect of osmotic stabilizers and concentration

In intact plant cells the mechanical protection of the cell wall prevents the disintegration of the cells when they are suspended in pure water or diluted media. Unless an osmotic stabilizer is used, the cytoplasmic membrane of the protoplast will disintegrate when the cell wall is digested. Therefore, protoplast isolation has been carried out in media of comparatively high osmotic pressure for maintaining the structural integrity. Moreover, under the same conditions for protoplast release, substances used for maintenance of protoplasts usually affect the final yield. $MgSO_4$ has been used for protoplast isolation from many species, including yeasts and moulds (Gascon and Villanueva, 1968). Pythium (Sietsma and de Boer, 1973), Schizophyllum commune (de Vries and Wessels, 1972) and Volvariella volvacea (Santiago, 1982).

Other salts such as NH_4Cl, KCl and NaCl are often used by many workers (Villanueva and Garcia-Acha, 1971). In organic compounds, mannitol and sorbitol were commonly used. In general cases, sugar and sugar alcohols were more effective with yeasts such as Mucor species (Ohnuki et al., 1982). On the other hand, inorganic salts have been proved to be more effective with filamentous fungi. In Volvariella, using $MgSO_4$ or KCl could yield 40% more protoplasts compared to sugar alcohols (Santiago, 1982). Organic stabilizers strongly inhibited protoplast isolation in Pythium (Sietsma and de Boer, 1973). In Pleurotus, $MgSO_4$ was by far the best of the stabilizers tested but only gave more than 16% in yield than mannitol. Factors involved in differences in such effectiveness were still not known (Peberdy, 1979).

The suitable concentration of rhamnose, used as the osmotic stabilizer varied largely from 0.5 M (Eddy and Williamson, 1957 and 1959) for Saccharomyces carlsbergensis and S. cerevisiae, to as high as 2.0 M for S. mellis (Weinberg and Orton, 1965). Such a variation might be due to the differences in internal osmotic pressure in different species. With the inorganic salt $MgSO_4$, 0.6 M was the suitable molarity for Pleurotus protoplast isolation. Concentrations higher or lower than 0.6 M seemed to be hypotonic or hypertonic to the osmotic pressure of the cytoplasm.

The protoplasts showed distinctive feature with large vacuoles in the cytoplasm when $MgSO_4$ was used in the lytic solution. Such a phenomenon which could have been caused by the presence of $MgSO_4$ was also found in protoplasts of Schizophyllum commune (de Vries and Wessels, 1972), Aspergillus nidulans (Peberdy and Isaac, 1976), and Volvariella volvacea (Santiago, 1982). This property was thought to provide a convenient separation of protoplasts from the hyphal wall residues by low-speed centrifugation. Moreover, using $MgSO_4$ instead of organic compounds seemed to prevent microbial contaminants during the isolation and culture of protoplasts (Gascon and Villanueva, 1965).

(c) Effect of pH

In general, enzymes have a characteristic optimum pH for their maximal activity. Small changes in pH can make a great difference in the rate of enzyme-catalyzed reactions. Protoplast release is optimal at pH between 5.8 and 6.5 in most systems (Gascon and Villanueva, 1965; de Vries and Wessels, 1972; Hamlyn et al., 1981; Ohnuki et al., 1982). However, the suitable pH value for Pleurotus was at 5.0. The result might be due to the interaction between the lytic enzymes and the specific cell wall components of Pleurotus.

(d) Effect of mycelial age

Mycelial age is an important factor determining the protoplast yield in fungi including yeasts (Duell et al., 1964; Brown, 1971; Shahin, 1972) and filamentous fungi (Peberdy, et al., 1976; Santiago, 1982). It is necessary to use young culture at the linear phase of growth for high protoplast yield. Results from these experiments have shown that mycelia or cells at early growth phase were more susceptible to the lytic enzymes than those from older cultures. Maximum protoplast yield was obtained from Pleurotus mycelia at the very initial stage of exponential growth. This suggested that the resistance against lytic enzyme developed rapidly in the early stages of growth.

(e) Effect of pretreatment

Pretreatment of mycelia or cells with thiol compound prior to digestion with lytic enzymes has been found to be more efficient or necessary with many fungi (Davies and Elvin, 1964; Sommer and Lewis, 1971; Dooijewaard Kloosterizel et al., 1973; Sietsma and de Boer, 1973; Hamlyn et al., 1981). It was suggested that the action site of such a compound was on the cell wall and not on the lytic enzymes (Davies and Elvin,1964). Another report showed that these compounds had the effect on the reduction of disulphide bonds in proteins which covered the wall surface allowing the entrance of the lytic enzymes

(Anderson and Millbank, 1966). However, the pretreatment was not necessary for effective protoplast isolation when very young mycelia were used (Sietsma and de Boer, 1973). From experiment with Geotrichum candidum, a better result was also obtained from young mycelia than from older ones and 2-mercaptoethanol showed a stronger stimulatory effect on protoplast isolation from old mycelia (Dooijewaard-Kloosterziel et al., 1973). The enhancement of protoplast yield by thiol compound and the resistance to lytic enzymes in older culture might be due to the modification in the cell wall. Therefore, the effect of 2 - mercaptoethanol on Pleurotus protoplast isolation was possibly due to the action on the protective layer covering the cell wall.

II. Reversion of Protoplasts

There have been numerous reports on the morphological study of protoplast reversion in fungi. The patterns of morphological development in protoplast reversion in Pleurotus was similar to other filamentous fungi such as Neurospora crassa (Bachmann and Bonner, 1959), Fusarium culmorum (García-Acha et al., 1966), Aspergillus nidulans (Peberdy and Gibson, 1971), Pythium (Sietsma and de Boer, 1973), Penicillium chrysogenum (Anne et al., 1974), Schizophyllum commune (de Vries and

Wessels, 1975) and Volvariella volvacea (Santiago, 1982).

Successful reversion on agar medium required a suitable osmotic stabilizer and nutrients. In Pythium, inorganic salts such as NaCl, $MgSO_4$, KCl and NH_4Cl could completely inhibit protoplast reversion and only organic compounds were suitable (Sietsma and de Boer, 1973). $MgSO_4$ also had inhibitory effect on Geotrichum candidum protoplast reversion (Dooijewaard et al., 1973). In contrast, inorganic salts can be used as osmotic stabilizers in many species including Penicillium chrysogenum (Anne et al., 1974) and Volvariella volvacea (Santiago, 1982). Studies with Fusarium culmorum showed that reversion frequency was influenced by the carbon source in the medium (Lopez-Belmonte et al., 1966). In Pythium, glucose was essential for reversion and nitrogen source was not necessary (Sietsma and de Boer, 1973). Yeast extract in the medium was not essential but it accelerated reversion in Penicillium (Anne et al., 1974).

There was no great difference in the reversion frequency in Pleurotus with complete and minimal media, but the presence of yeast extract and peptone seemed to stimulate the vigorous growth of mycelia after protoplast reversion. However, the reversion frequency in Pleurotus was exceedingly low and only 4-6% was obtained when compared to other species such as Fusarium culmorum (Lopez-Belmonte et al., 1966), Geotrichum candidum

(Dooijewaard-Kloosterziol, et al., 1973), Pythium (Sietsma and de Boer, 1973), Penicillium chrysogenum (Anne et al., 1974) and Schizophyllum commune (de Vries and Wessels, 1975). In the several latter species, the reversion frequency varied from 20-80%. The absence of a nucleus might be one obvious factor that caused the variation in reversion frequency (Garcia-Acha et al., 1966). Since there are no further reports on the protoplast reversion in other edible mushrooms, it is not known whether such a low reversion frequency is a general phenomenon in these fungal species. Up to date, there have been only a few reports on the protoplast isolation and reversion in Basidiomycetes including Schizophyllum commune (de vries and Wessels, 1972, 1973 and 1975), Coprinus lagopus (Moore, 1975), Volvariella volvacea (Santiago 1982a and 1982b), and Lentinus (Qiu, et al., 1982). More work is still needed to be done on the protoplast technology in Basidiomycetes, especially in edible mushrooms.

CHAPTER 6

INTERSPECIFIC PROTOPLAST FUSION BETWEEN *PLEUROTUS SAJOR-CAJU* AND *P. FLORIDA*.

A. INTRODUCTION

I. Role of Protoplast Fusion and its Requirements

With the development of efficient enzymatic methods for protoplast isolation in fungi and higher plants, many plant breeders have thought that somatic cell fusion can overcome the restriction on gene(s) flow between organisms with vegetative or sexual incompatibility (Schenk and Hildebrandt, 1968; Peberdy, 1979; Cocking et al., 1981). In fact, isolated protoplasts possess an important property of being able to take up organelles including chloroplasts (Bonnett and Erikson, 1974) and nuclei (Potrykus and Hoffmann, 1973), viruses (Cocking, 1966) and even bacteria (Davey and Cocking, 1972). Therefore, isolated protoplasts can be utilized for the incorporation of desirable genetic information into plant cells. More importantly, interspecific protoplast fusion could show a great potential as an aid in the genetic manipulation of agricultural and industrial strains. However, one of the

most important practical uses of isolated protoplasts is for somatic hybridization, especially in sexually incompatible plants and in cases where conventional methods of breeding fail to operate. The somatic hybrids, containing the combined properties of different strains or species, provide the opportunity for geneticists to study chromosomal homology between species, and taxonomical relationships. The principal requirements for the successful processes in protoplast fusion are as follow: (1) it is essential that the membranes of two different protoplasts must be brought into contact for fusion; (2) the two nuclei of the heterokaryons must fuse or both nuclei in the hybrid protoplast must be able to proceed mitotic divisions simultaneously; and (3) the resulting hybrid protoplast must be able to regenerate and revert into normal organism.

II. Methods for Protoplast Fusion

In recent years, a number of methods have been developed for accomplishing protoplast fusion. Ultrastructural observations have revealed that the two protoplasts adhered to each other and then the membranes fused in the small contact areas followed by the intermingling of the cytoplasm of the two protoplasts (Withers and Cocking, 1972; Burgess and Fleming, 1974).

It was observed under the electron microscope that protoplasts which were released from the adjoining cells, could sometimes fuse through the plasmodesmata to form a large multinucleate protoplast (Withers and Cocking, 1972). Such a spontaneous fusion was strictly intraspecific and only occurred occasionally. Therefore, many workers have to employ a variety of techniques, using different fusogens and procedures to achieve protoplast fusion in order to obtain interspecific somatic hybrids. Power and co-workers (1970) successfully demonstrated the intra- and interspecific fusion between protoplasts obtained from the root tips of maize and oat seedlings using $NaNO_3$ (0.25 M) as the fusogen. But they could not observe any adhesion of the isolated protoplasts when the protoplasts were only suspended in sucrose solution for 1 hr. Kameya, from his experiment (1973), found that gelatin and the early products of its degradation at a concentration of 2.5% could induce aggregation of protoplasts isolated from Allium fistulosum, Brassica chinensis and Daucus carota at a high frequency within 1 hr. Hartmann and his co-workers (1973), applied the immunological method and succeeded in obtaining the agglutination of protoplasts from Bromus, Glycine and Vicia and found that Glycine and Bromus antibodies could cross-react and agglutinate

with Vicia protoplasts. Also, these protoplasts were viable and could undergo cell division after the antibody treatment. A wide range of additives such as poly-L-ornithine, poly-D-lysine, poly-L-lysine, concanavalin A, cytocholasin and protamine sulphate (Grout and Coutts, 1974), glycerol and dimethyl sulphoxide (Ahkong et al., 1975) have also been used.

Among the fusogens employed so far, PEG seems to be the most promising one because it has the great advantages of being simple to use and gives a high frequency of fusion. It is highly soluble in water. The general formula of PEG is $HOCH_2-(CH_2-O-CH_2)n-CH_2OH$, and its molecular weight ranges from less than 1,000 to over 20,000 (Meyer, 1952). It has been suggested that the ether linkages in the PEG make the molecule slightly negative in polarity which is capable of forming hydrogen bonds with the positively polarized groups of proteins, carbohydrates, etc. The enhancing adhesion by the addition of Ca++ could be due to the formation of a Ca++-bridge between the negatively polarized groups of membraneous proteins and PEG (Kao and Michayluk, 1974). The use of PEG in the protoplast fusion was established independently by two research groups (Kao and Michayluk 1974; Wallin et al., 1974). From their study, Kao and Michayluk made the following conclusions: (1) no hybrid

was detected when PEG was not used; (2) the PEG concentration was very important for high fusion frequency, e.g. 0.2 M was much better than 0.1 M; (3) there was no significant difference in the effect of PEG of MW 1,300 - 1,600 and PEG of MW 6,000 - 7,500 on the fusion frequency; (4) the fusion frequency was enchanced greatly from 6.2% to 9.0% when Ca++ was added in the PEG solution; and (5) a prolonged incubation of protoplasts in PEG solution could reduce the fusion frequency because many protoplasts were killed. Anne and Peberdy (1975), based on their experimental results, established the conditions required for producing a high frequency of viable heterokaryons in fungal protoplasts fusion. They found that the supplement of PEG solution with Ca++ and the adjustment of high pH (e.g. pH 9.0) gave the best result. If K+, Na+, or Mg++ were present, these ions could be preferentially linked to the membranes and be competitive with Ca++ which was reflected in the fusion yield. Peberdy (1979), also emphasized that the concentration of PEG was critical for fungal protoplast fusion. In general, 30% PEG was the optical concentration, a concentration below 20% would cause protoplast lysis while a concentration higher than 30% was toxic to protoplasts and would cause them to shrink. PEG used under above conditions , has also been proved to be a very effective

chemical for fusion in mammalian cells (Ahkong et al, 1975), higher plant protoplasts (Kao and Michayluk, 1974), fungal protoplasts (Anne and Peberdy, 1976; Ferenczy et al., 1974, 1975 and 1976) and bacterial protoplasts (Fodor and Alzoldi, 1976; Schaeffer et al., 1976). More recently, high fusion frequency in plant protoplasts has been obtained by employing electrofields (Zimmermann and Scheurich, 1981). During the dielectrophoretically induced adhesion among protoplasts, the fusion of protoplasts could proceed under physiological pH conditions without the use of chemical compounds.

III. Selection of Regenerated Fusion Products of Protoplasts

With efficient fusion techniques available, the focus has been concentrated increasingly on the development of selection methods for somatic hybridization. Selection of regenerated fusion products requires a demonstration of genetic contribution from both parents. If the parental strains have distinctive morphological or biochemical differences involving nuclear and cytoplasmic markers, the selection systems could make it possible to isolate the somatic hybrids on the culture medium. At present, techniques available for the recognition of fusion products can be grouped under the

following categories: (1) genetic, (2) phenotypic, (3) biochemical, and (4) physical. Nutritional complementation between auxotrophic mutants is a most popular method for selecting the fusion products in microbial protoplast fusion. Since the auxotrophic mutants usually behave in a recessive manner, stringent selection pressure on the minimal medium could be applied to select for complementing prototrophic hybrids. On the other hand, a careful check of back-mutation or cross-feeding, and all other necessary control also have to be included in the experiment.

Complementing auxotrophic markers have been found to be suitable even in cases when back-mutation frequency was 10^{-5} (Sidorov and Maliga, 1982). It is important, though, that the isolated putative hybrids should also be subjected to genetic and biochemical analysis in order to verify the genetic constitution of the isolates. Nevertheless, this procedure is unfavourable for industrial strain improvement programmes because the introduction of auxotrophic mutation in strains often impairs certain metabolic pathway(s).

Drug- or heavy metal-resistant gene(s) could be an ideal genetic marker (Cocking et al., 1981). In fact, the use of drug-resistant markers such as oligomycin-resistance (Rowlands and Turner, 1973) has been applied

for screening industrially important strains. Mitochondrial mutants with antibiotic-resistance were also suggested as good markers in protoplast fusion with industrial yeast strains (Spencer et al., 1980). In Saccharomyces, respiratory-deficient petite mutants which fail to grow on non-fermentable substrates such as glycerol have the following advantages in protoplast fusion. These petite mutants, being the result of mutation of mitochondrial DNA, can avoid the deletion of nuclear genes which encode the functions associated with the fermentation process.

The application of non-selected markers, such as colony morphology, for selection requires much effort to isolate the fusion products. However, the visual identification and manual isolation of fusion products is one of the most popular selection techniques for higher plants in the past. By careful choice of the protoplast origin, visible markers are readily available practically from different plant organs and plant species. Fusion of leaf mesophyll protoplasts which have chlorophyll or other pigments, with protoplasts of cultured cells which usually contain starch granules has been used as a general combination of visual markers. Such characteristics have been shown to allow the manual isolation of visually identified fusion products and subsequent culture in a

microdroplet (Power et al., 1970 and 1976; Kao, 1977; Gleba et al., 1982; Hein et al., 1983). An alternative is to use artificial visual markers, and this involves labelling parental protoplasts separately with different fluorescence stains (e.g. rhodamine B, flouorescein, isothiocyanate). Fusion products which showed different fluorescence stain could be manually isolated (Patnaik et al., 1982). However, this very labourious method requires further careful confirmation of the putative hybrids (Binding et al., 1982).

Biochemical inactivation with specific inhibitors could serve as a means of selective pressure against parental protoplasts. Wright (1978) used different SH-group inhibitors for simultaneous inactivation of both parents in hybrid selection. Harms and Potrykus (1978) developed a method for the physical separation of fusion products by centrifugation of the PEG-treated protoplast mixture in an iso-osmotic density gradient system. This resulted in a 2- to 7-fold relative enrichment hetero-karyocytes which had intermediate density when compared to that of parental protoplasts.

IV. Fungal Protoplast Fusion

The controlled fusion of fungal protoplasts was first achieved by Ferenczy and co-workers (1974) through the nutrient complementation between auxotrophs of

Geotrichum candidum which does not have anastomosis and heterokaryons or sexual processes. However, a high frequency of protoplast fusion could not be obtained in the absence of fusogen. The average frequency of complementation was 1.6×10^{-6}. Since then, both intra- and interspecific hybridizations through protoplast fusion have been achieved in yeasts and filamentous fungi. Intraspecific protoplast fusion appears to be the only means by which hybridization for strain improvement can be achieved for fungi especially in yeasts in which sexual conjugation and genetic recombination are not observed. Such technique can also be applied to the breeding of industrial strains of the usually polyploid or aneuploid Saccharomyces cerevisiae which has a low frequency of sporulation and produces spores with poor viability (Stewart, 1981). Another advantage of using protoplast fusion in yeasts is that the mating-type barrier can be eliminated and the fusion products will be homozygous for the mating-type gene(s). In Saccharomyces cerevisiae, the diploids homozygous for the mating types were genetically stable and regularly non-sporulating (Stewart, 1981). By this method, it is possible to produce not only diploids but also polyploids homozygous for the mating-types which may have industrial advantages. Intraspecific hybridization by protoplast fusion has been achieved in

many yeasts including Candida tropicalis (Fournier et al., 1977), Hansenula wingei (Svoboda, 1978), Kluyveromyces lactis (Allmark et al., 1978), Saccharomyces cerevisiae (Ferenczy and Maraz, 1977, van Solingen and van der Plaat, 1977), Saccharomycopsis lipolytica (Stahl, 1978) Schizosaccharomyces pombe (Sipiczki and Ferenczy, 1977b; Svoboda, 1978), and Saccharomyces diastaticus (Spencer et al., 1980). The first successful interspecific protoplast fusion in yeasts was obtained from the hybridization between Kluyveromyces fragilis and K. lactis auxotrophic mutants (Whittaker and Leach, 1978). In contrast, the hybrids obtained from the interspecific protoplast fusion between Schizosaccharomyces pombe and S. octosporus were very unstable and segregated rapidly resulting in colonies with characteristics of only the S. octosporus parent (Sipiczki, 1979). Intergeneric hybridization has also been achieved by protoplast fusion between auxotrophic mutants of Candida tropicalis + Saccharomyces fibuligera (Provost et al., 1978) and Saccharomyces strains + Kluyveromyces lactis (Stewart, 1981).

In filamentous fungi, vegetative or heterokaryotic incompatibility has been demonstrated in a number of species (Esser and Blaich, 1973). The hyphae of vegetatively compatible strains regularly fuse together to form normal heterokaryons whereas incompatible strains

are unable to carry out the hyphal fusion. In such a case, the barrier due to vegetative incompatibility could be overcome by means of protoplast fusion. For example, heterokaroytic formation among Aspergillus niger strains was only obtained occassionally through mycelial fusion and could be considered insignificant. There was still a very low fusion frequency when regenerated protoplasts were used instead of the non-regenerated ones. But when protoplast fusion was used , a complementation frequency of 2.5×10^{-3} could be obtained (Ferenczy et al., 1977). Croft and Dales (1979) succeeded in using the diploid strain selected through protoplast fusion to study the location of the het-gene in Aspergillus nidulans. They found that haploidization analysis of vegetative diploids, produced from fusing incompatible strains,provided a rapid method for genetic analysis when compared to the conventional analysis of sexual progenies. Interspecific protoplast fusion in filamentous fungi has been achieved in Aspergillus, Penicillium and Mucor species. Based on several reports, the possibility of interspecific hybridization and the viability of the heterokaryons mainly depended on species relationship and the nuclear/cytoplasmic compatibility. In the experiment carried out by Peberdy and co-workers (1977), slow-growing interspecific heterokaryons were isolated on the

minimal medium following the fusion between closely related species Penicillium chrysogenum + P. cyaneofulcum. Prolonged cultivation of these heterokaryons gave rise to hybrids which were distinctive in comparison to the parental strains. When these hybrids were cultured on complete medium containing fluorophenylalanine or benomyl, the stability of heterokaryons were broken down and several different prototrophic and auxotrophic colony types were obtained. Similar results were also obtained in the protoplast fusion experiment using the closely related Aspergillus nidulans and A. rugulosus (Kevei and Peberdy, 1977).

Anne and coworkers (1976) obtained heterokaryons from protoplast fusion between less related Penicillium species, P. chrysogenum + P. roqueforti, which were quite different in their morphology and behaviour. In their experiment, they obtained three main types of the interspecific hybrids which were morphologically different from the parental strains. The type 1 and type 2 colonies were prototrophic and could produce penicillins as the parental P. chrysogenum did, but produced parental P. roqueforti spores. The type 3 colonies could produce penicillins but relaesed prototrophic spores. The heterokaryons sometimes showed sectors of haploid P. roqueforti segregants only, thus P. chrysogenum could not be re-isolated. In the protoplast fusion between

A. nidulans + A. fumigaus, only A. nidulans type segregants could be recovered among the interspecific heterokaryons (Ferenczy et al., 1977). It was common that reisolation of one parental strain failed in heterokaryons which produced from two less related species. Not all protoplast fusion between less related species could produce viable interspecific heterokaryons. According to Anne's report (1983), very small colonies developed when Penicillium chrysogenum was used to fuse with P. cyclopium, with P. lanosum or with P. nigricans, and these colonies could not be subcultured on minimal medium. Furthermore, no fusion products could be recovered from protoplast fusion among P. chrysogenum + P. raistrickii, P. roqueforti + P. nigricans, or P. roqueforti + P. patulum. These results implied that a strong imbalance of the nuclear constitution or total incompatibility occurred among these species. In contrast to the results in Penicillium, only closely related species of Aspergillus will form interspecific hybrids. The formation of fusion products have consistently failed when A. nidulans was used to fuse with A. terreus, A. versicolor, A. amsteldami or A. niger. Even when the species which belong to the 'nidulans' group including A. unguis, A. stellatus and A. heterothallicus were used to hybridize with A. nidulans, somatic hybrids could not be obtained (Croft ans Dales, 1983). It was suggested that, in such cases, mitochondrial incompatibility might be involved in the failure to form

interspecific heterokaryons.

V. Isoenzymes as Biochemical Markers

The term isoenzyme , first introduced by Markert and Moller (1959), was defined as an enzyme which has multiple molecular forms with similar or identical substrate specificity in the same organism. Furthermore, Markert (1968) has suggested that one might modify the term isoenzyme with such adjectives as allelic, nonallelic, conformational, hybrid, etc. for more precise definition. Isoenzymes may differ in their primary structure because they can be encoded in different genes, either allelic or nonallelic. In many cases, the primary structure could be further modified by conjugation of the molecules (e.g. the ions) with the reactive groups such as amino, carboxyl, or hydroxyl group of the amino acid components of the polypetide structure. It is well known that the great variation detected in isoenzymes is due to the genetic bases which code them. Thus, this property of isoenzymes render them as useful markers in a number of biological investigations (Scandalios, 1968). Although some conventional markers are available and have been used in the studies with somatic cell hybridization, many of them, such as morphological markers, can be detected only after differentiation of the organ or the tissue. Isoenzymes

could be ideal and natural markers for somatic cell genetic studies and for several breeding programmes in many respects: (1) the abundance of naturally existing variants in plant population obviating the necessity for mutant induction (Shannon, 1968; Scandalios, 1974); (2) markers are often expressed in the undifferentiated state of cell culture; (3) the ease of detection; and (4) the applicability to small amounts of material.

Moreover, the genetic basis for isoenzymes has been studied in detail (Schwartz, 1964 and 1966; Scandalios, 1969; Schwartz et al., 1965; Schwartz and Endo, 1966; Moess et al., 1974). The zymogram technique involves electrophoretic separation of chemical components in crude extract from the tissue or the culture. This is demonstrated by band distribution of enzyme activity using specific histochemical staining of electrophoretic medium such as starch gel or acrylamide gel. The technique allows for the resolution of isoenzymes mainly on the basis of electrical charges and molecular size differences (Hunter and Markert, 1957). A number of closely related clones and all hybrids tested have been distinguished by this method in potato (Desborough and Peloquin, 1968), Nicotiana species (Lo Schiavo et al., 1980) and citrus (Iglesias et al., 1974; Button et al., 1976; Torres et al., 1978). Moreover, analyzing band patterns of isoenzymes from roots in order to distinguish between

nucellar and zygotic seedlings has now become a routine work in citrus breeding programme (Ben-Hayyim et al., 1982). As protoplast fusion can now be successfully performed on plant cells, different isoenzyme patterns in both parents could provide a powerful tool to identify somatic hybrids at the earliest possible stage as shown by the work done on soybean-Nicotiana somatic hybid cell lines (Wetter, 1977), Datura (Lonnendonker and Schieder, 1980), and Nicotiana species (Lo Schiave et al., 1980).

The breeding of several cultivated mushrooms is currently important but difficult (Elliott, 1982). The only available means of isolating hybrids is by the use of morphological characters or auxotrophic mutants, but there are many drawback with using these genetic markers. Morphological markers require distinctive and stable expressions in the vegetative mycelia or in the fruit bodies, but most morphological phenotypes are considerably influenced by environmental factors and thus are unreliable. The use of auxotrophic mutants can overcome many difficulties associated with the use of morphological characters, but such isolates often have deficiency in certain biochemical pathway(s), thus rendering them limited in their use for practical breeding programmes (May and Royse, 1981). The electrophoretic methodology has been established for the recognition of new lines by isolation from natural populations and also hybri-

dization through nuclear exchange (Boisselier-Dubayle, 1983; May and Roy e, 1982; Royse and May, 1982a and 1982b).

B. RESULTS

I. Protoplast Fusion

A mixture of PEG-pretreated protoplasts from Pleurotus sajor-caju and P. florida mutants , (Su223 + Ful56), were plated on both complete and on minimal media. The protoplasts on the complete medium reverted well after 5 - 6 day incubation while those on minimal medium could develop only sparse, small and slow prototrophic mycelial colonies even after 8 - 10 day incubation. After one more week incubation, different forms of colonies could be observed on the complete medium (Fig. 6.1). This indicated that these colonies could have developed from a mixture combination of non-fused protoplasts, fusion protoplasts from the same parental strain, and fusion products of protoplasts from different parental strains. The lower reversion frequency of protoplasts on the complete medium after PEG treatment, when compared to that of non-PEG treatment, might be partly due to the damage effect of PEG on the protoplasts (Table 6.1, 6.2, and 6.3). When a mixture of the protoplasts of two parental mutants (e.g., Su223 and

Fig. 6.1 Protoplast reversion of Pleurotus auxotrophs on osmotic medium after PEG treatment. The plating protoplasts were incubated at 28°C for 12 days.

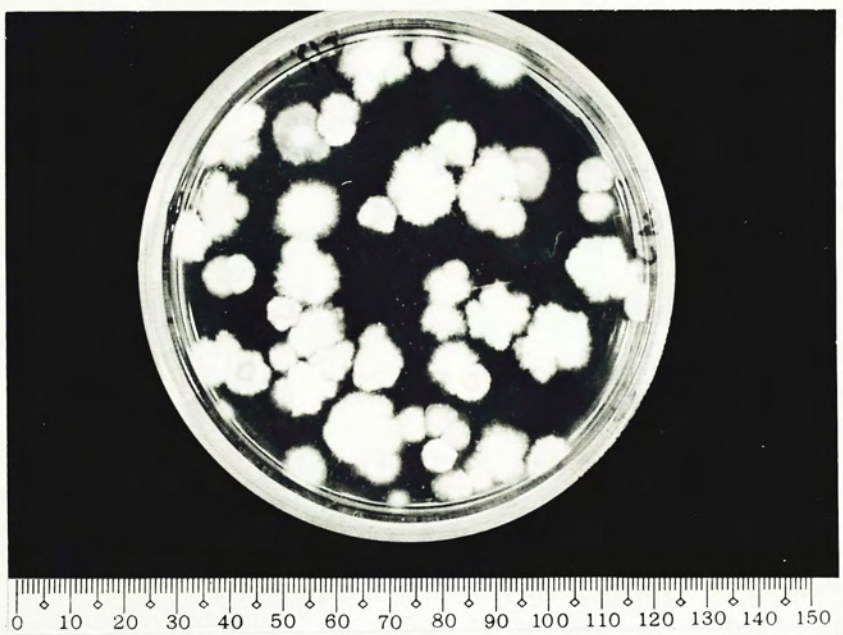

0 10 20 30 40 50 60 70 80 90 100 110 120 130 140 150

Table 6.1 Reversion frequency of polyethylene glycol (PEG) treated protoplast mixture of Su223 and Fu156 of Pleurotus. Non-PEG treatment served as a control.

Protoplast reversion on	PEG treatment		Non-PEG treatment	
	No. of colony per plate	Reversion freq.(%)	No. of colony per plate	Reversion freq.(%)
CM	63.5 ± 7.89*	3.39	119.7 ± 21.8	6.4
MM+glutamic acid	28.0 ± 3.08	1.49	NT	NT
MM+riboflavin	24.3 ± 1.30	1.30	NT	NT
MM	1.7 ± 0.88	0.09	0.0	0.0
Fusion frequency= 2.69 %				

Note: *Standard deviation,
Su223=P. sajor-caju mutant requiring glutamic acid,
Fu156= P. florida mutant requiring riboflavin,
CM=Complete medium,
MM=Minimal medium,
NT=Not tested.

Table 6.2 Reversion frequency of polyethylene glycol (PEG) treated protoplast mixture of Su42 and Fu22 of Pleurotus. Non-PEG treatment served as a control.

Protoplast reversion on	PEG treatment		Non-PEG treatment	
	No. of colony per plate	Reversion freq.(%)	No. of colony per plate	Reversion freq.(%)
CM	60.0 ± 6.7*	2.83	87.7 ± 4.2	4.14
MM+folic acid	24.3 ± 3.3	1.15	NT	NT
MM+cytosine	22.7 ± 3.6	1.07	NT	NT
MM	2.6 ± 0.5	0.12	0.0	0.0

Fusion frequency= 4.33 %

Note: *Standard deviation
Su42=P. sajor-caju mutant requiring folic acid,
Fu22=P. florida mutant requiring cytosine,
CM=Complete medium,
MM=Minimal medium,
NT=Not tested.

Table 6.3 Reversion frequency of polyethylene glycol (PEG) treated protoplast mixture of Su42 and Fu156 of *Pleurotus*. Non-PEG treatment served as a control.

Protoplast reversion on	PEG treatment		Non-PEG treatment	
	No. of colony per plate	Reversion freq.(%)	No. of colony per plate	Reversion freq.(%)
CM	39.7 ± 6.2*	2.48	54.5 ± 3.5	3.41
MM+folic acid	21.5 ± 2.7	1.43	NT	NT
MM+riboflavin	21.0 ± 3.2	1.31	NT	NT
MM	4.8 ± 5.5	0.30	0.0	0.0
Fusion frequency= 12.08 %				

Note: *Standard deviation,
Su42=*P. sajor-caju* mutant requiring folic acid,
Fu156= *P. florida* mutant requiring riboflavin,
CM=Complete medium,
MM=Minimal medium,
NT=Not tested.

Ful56) were treated without PEG, mycelial colonies could develop only on the complete medium but not on the minimal medium. Mycelial colonies were not observed on the minimal medium when protoplasts of each parent were treated with PEG before plating. These results indicated that the prototrophic colonies on the minimal medium were the outcome by auxotrophic complementation after protoplast fusion between Su223 + Ful56, Su42 + Fu22, and Su42 + Ful56 and not the products of back- mutation or cross-feeding between non-fused protoplasts. The fusion frequencies were 2.69% 4.32% and 12.1% for protoplast fusion between Su223 + Ful56, Su42 + Fu22 and Su42 + Ful56 respectively.

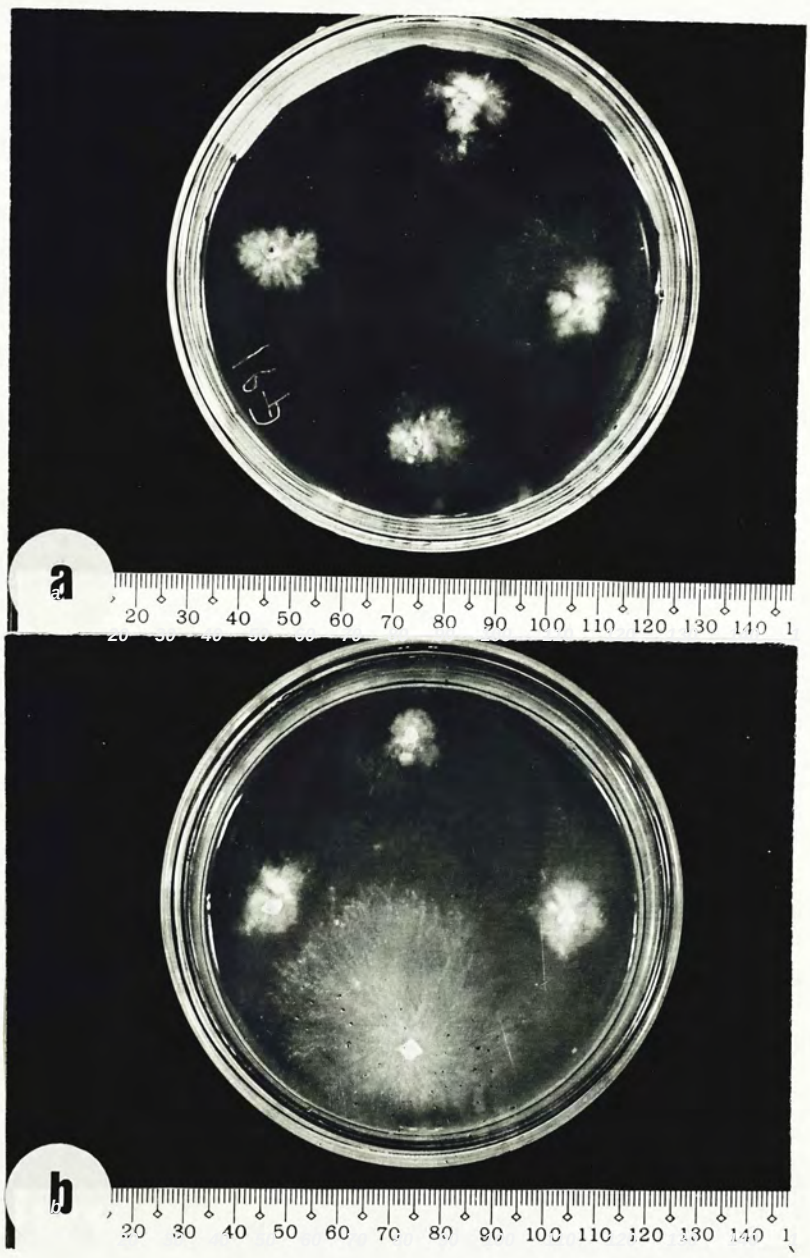
II. Stability and Growth Rate of Heterokaryons on the Complete and Minimal Media

The interspecific heterokaryons of Su223 + Ful56 were obtained in the first successful experiment of protoplast fusion. After subculture on a fresh minimal medium, the heterokaryons grew slowly forming only sparse colonies which were regular in shape (Fig. 6.2). It was noted that the mycelial growth of isolate SF1-10 was much faster than other isolates. The external morphology of the colonies were quite similar to that of mutant Su223. There was no distinctive difference in the morphology among all these

Fig. 6.2 Comparison of mycelial colony morphology among isolates of interspecific heterokaryon (SF1) of Pleurotus. Clockwise from top.

(a) SF1-5, SF1-8, and SF1-7.

(b) SF1-9, SF1-12, SF1-10 and SF1-11.


a
20 30 40 50 60 70 80 90 100 110 120 130 140
b
20 30 40 50 60 70 80 90 100 110 120 130 140

isolates. On the complete medium, the heterokaryons seemed to be stable and no distinguishable segregation of mycelial growth could be observed (Fig. 6.3).

Growth rate of heterokaryotic isolates was indicated by the diameter of colonies formed. On the complete medium, colonies of different isolates varied very little in diameter, only from 5.1 cm to 6.4 cm, while those on the minimal medim showed a great variation in diameter. The isolates, SF1-4, SF1-5, and SF1-8, grew extremely slow. On the other hand, the growth rate of the isolates SF-1, SF1-3, and SF1-10 were comparable to those grown on the complete medium (Table 6.4). The morphology of SF2 isolates was similar to that of mutant Fu22. Two distinctive types of colonies could be observed when SF3 isolates were cultured on complete medium; one type was sparse and fast growing and the other was compact in growth (Fig. 6.4). The growth rate of interspecific heterokaryons of SF2 and SF3 was shown in Tables 6.5 and 6.6. To study the stability of interspecific heterokaryons after subculture, three inocula from different regions of the colony margin were subcultured on the complete medium. Again, there was no observable mycelial segregation and the growth rate of isolates was also comparable to that of the first complete medium culture.

Fig. 6.3 Comparison of growth rate of two isolates of interspecific heterokaryon (SF1) of Pleurotus on complete medium (left) and on minimal medium (right).

(a) Interspecific heterokaryon isolate, SF1-3.

(b) Interspecific heterokaryon isolate, SF1-8.

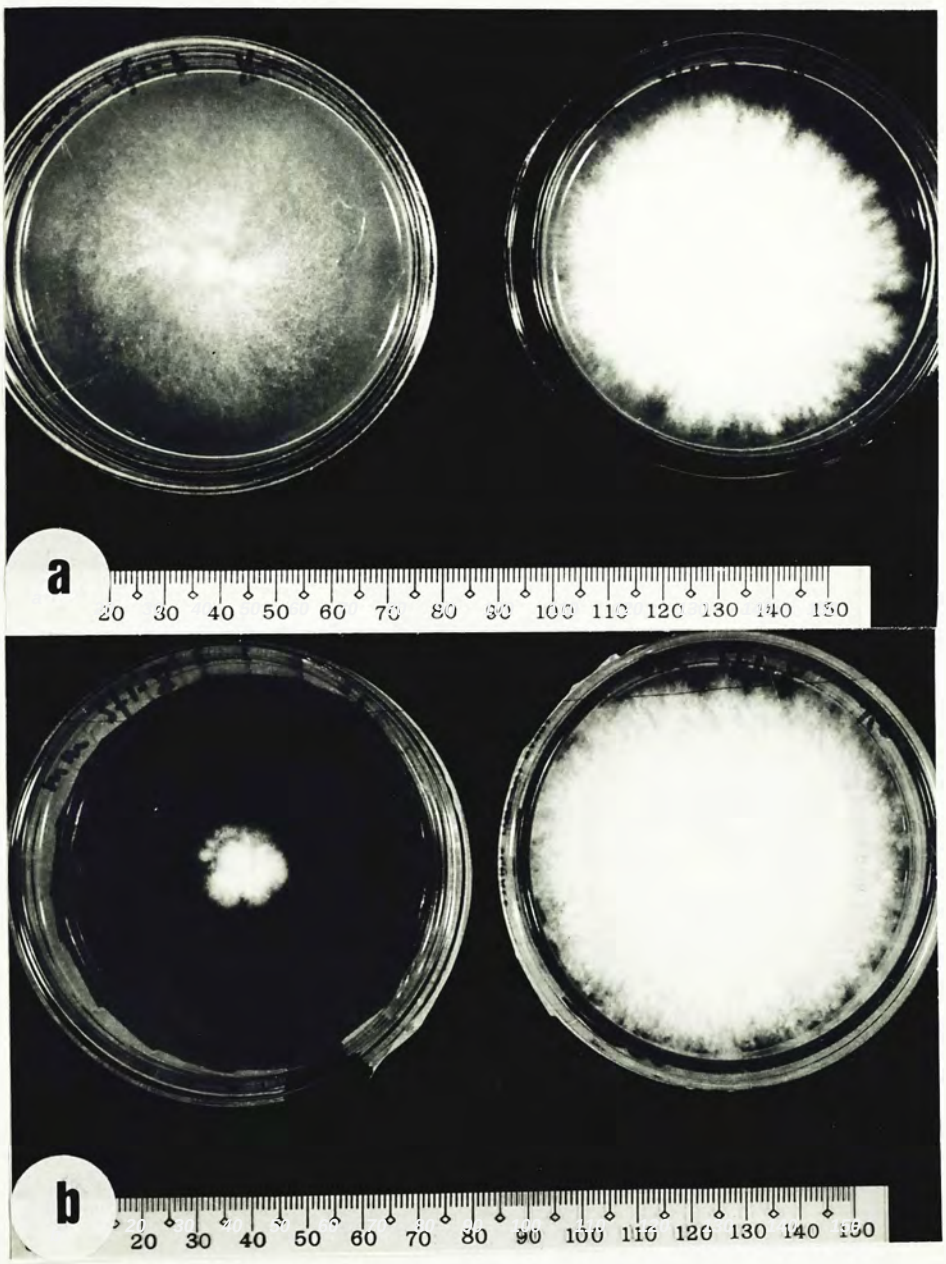

a
20 30 40 50 60 70 80 90 100 110 120 130 140 150
b
20 30 40 50 60 70 80 90 100 110 120 130 140 150

Table 6.4 Growth rate of interspecific heterokaryon (SF1) isolates of Pleurotus after 4-week cultivation.

Isolate	Growth rate: Colony diameter (cm)		
	Complete medium	Minimal medium	% *
SF1-1	6.30 ± 0.20**	5.83 ± 0.29	92.58
SF1-2	6.07 ± 0.25	2.77 ± 0.12	45.63
SF1-3	6.37 ± 0.40	6.10 ± 0.29	95.76
SF1-4	6.20 ± 0.24	1.63 ± 0.17	26.29
SF1-5	5.57 ± 0.68	1.77 ± 0.12	31.78
SF1-6	6.17 ± 0.31	4.10 ± 0.22	66.45
SF1-7	5.10 ± 0.25	1.80 ± 0.45	35.29
SF1-8	6.30 ± 0.22	1.77 ± 0.12	28.09
SF1-9	5.87 ± 0.46	2.17 ± 0.46	36.97
SF1-10	6.13 ± 0.25	5.87 ± 0.46	95.76
SF1-11	5.50 ± 0.33	3.50 ± 0.49	63.64

*Ratio of growth rate on minimal medium to that on complete medium.

**Standard deviation.

Fig. 6.4 Comparison of mycelial colony morphology among isolates of interspecific heterokaryon (SF3) of Pleurotus.

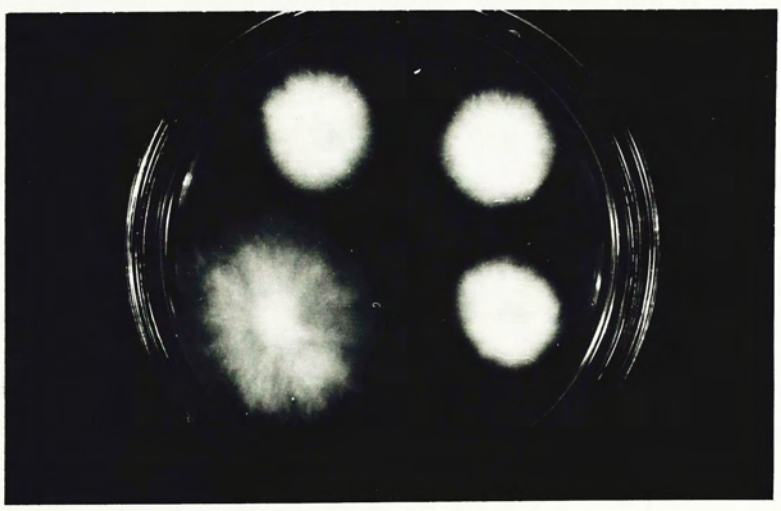


Table 6.5 Growth rate of interspecific heterokaryotic (SF2) isolates of Pleurotus after 2-week cultivation.

Isolate	Growth rate: Colony diameter (cm)		% *
	Complete medium	Minimal medium	
SF2-1	2.47 ± 0.21 **	1.50 ± 0.22	60.73
SF2-2	3.23 ± 0.17	1.77 ± 0.21	54.79
SF2-3	2.53 ± 0.17	1.63 ± 0.11	64.43
SF2-4	2.90 ± 0.22	2.20 ± 0.21	75.86
SF2-5	2.73 ± 0.15	1.73 ± 0.19	63.37

*Ratio of growth rate on minimal medium to that on complete medium.

**Standard deviation.

Table 6.6 Growth rate of interspecific heterokaryotic(SF3) isolates of Pleurotus after 4-week cultivation.

Isolate	Growth rate: Colony diameter (cm)		%*
	Complete medium	Minimal medium	
SF3-1	5.34 ± 0.16**	1.72 ± 0.09	32.21
SF3-2	4.13 ± 0.19	2.31 ± 0.24	55.93
SF3-3	6.50 ± 0.24	2.07 ± 0.22	31.85
SF3-4	3.81 ± 0.22	2.25 ± 0.29	59.06
SF3-5	1.64 ± 0.31	0.87 ± 0.12	53.05
SF3-6	1.43 ± 0.12	0.93 ± 0.12	65.03
SF3-7	4.46 ± 0.29	2.17 ± 0.26	48.65
SF3-8	6.03 ± 0.25	2.23 ± 0.21	36.98

*Ratio of growth rate on minimal medium to that on complete medium.

**Standard deviation.

III. Fructification of Interspecific Heterokaryons

For a study of the fruiting ability of the interspecific heterokaryons, small inocula were subcultured on the cotton waste in bottles. The mycelia grew very slowly and required about 4 weeks to cover the surface of the medium. In contrast, their dikaryotic parents took only 12-14 days to do so. After the dikaryotic mycelial cultures were transferred to moist and light conditions, pin-head like primordia could be observed within one week and these developed into young fruit bodies within another week. On the other hand, some heterokaryotic isolates of SF1, including SF1-1, SF1-5, SF1-6, and SF1-7 could form pin-head primordia (Fig. 6.5), but these could not differentiate into fruit bodies. Other isolates of SF1 and heterokaryons SF2 and SF3 did not form any pin-head like structure at all.

IV. Nuclear Staining of Interspecific Heterokaryons

To study the nuclear number in each cell, several staining dyes including Giemsa, aceto-orcein and carbol fuchsin were tested. It was found that Giemsa could give the best result for nuclear observation (Fig 6.6). Most cells of the SF1 heterokaryotic isolates were binucleate, except SF1-5 in which some cells were multinucleate (Table 6.7). All cells of the five

Fig. 6.5 Pin-head like structure (indicated by arrows) on interspecific heterokaryon (SF1-5) of Pleurotus after one-month culture. The culture was grown on cotton waste at 28°C.

Fig. 6.6 Cells in interspecific heterokaryon (SF1-5) of *Pleurotus* containing two or more than two nuclei per cell (indicated by arrows). Nuclei stained with Giemsa, magnification 1,000x.

(a) Binucleate cells.

(b) Multinucleate cells.

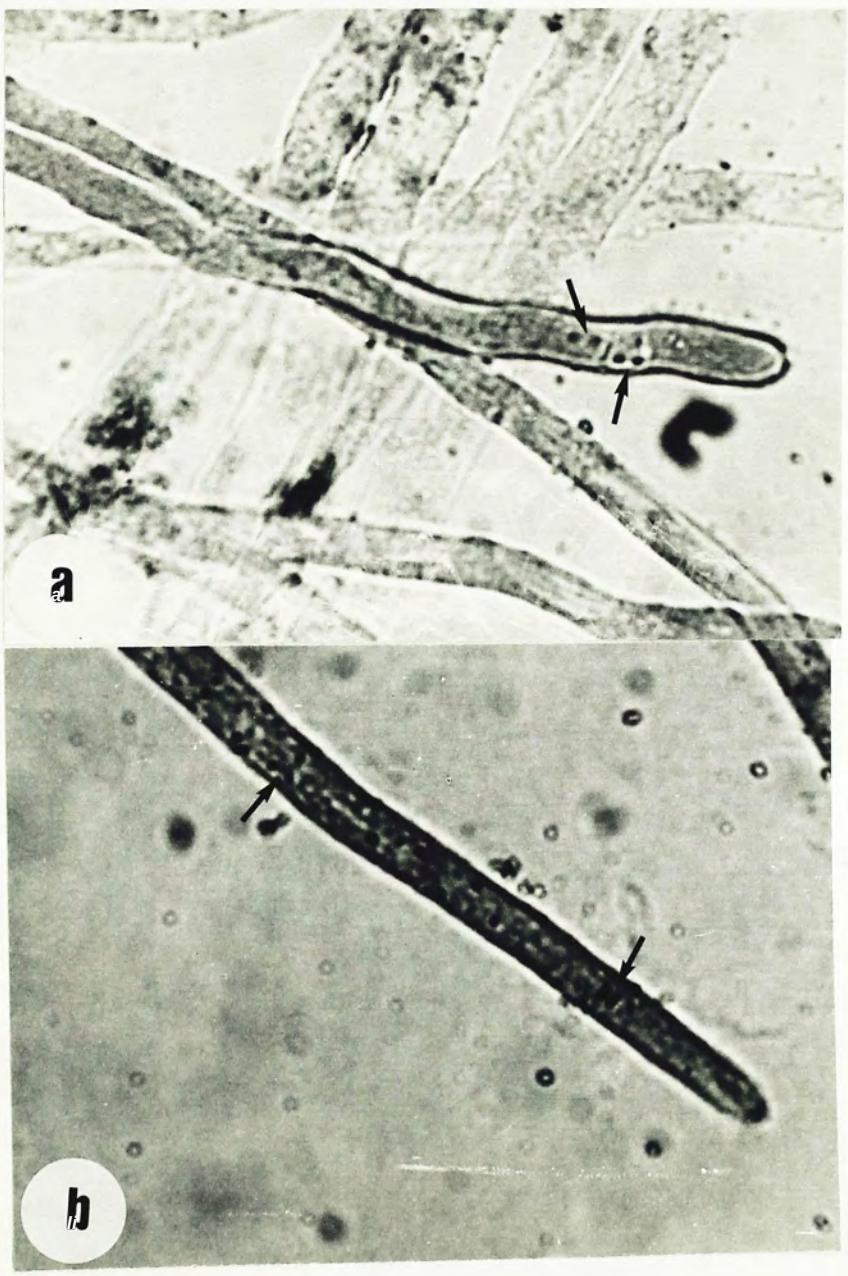

a
b

Table 6.7 Number of nuclei in apical cells and subapical cells of isolates of interspecific heterokaryon (SF1) of *Pleurotus*.

Isolates	Apical cell		Subapical cell	
	2	3-5	2	3-5
SF1-1	35	0	87	0
SF1-2	34	0	81	0
SF1-3	35	0	97	0
SF1-4	23	2	81	2
SF1-5	39	7	113	17
SF1-6	50	1	105	8
SF1-7	39	0	110	6
SF1-8	45	6	136	0
SF1-9	43	2	131	2
SF1-10	31	0	93	0
SF1-11	24	0	78	0

SF2 heterokaryotic isolates were binucleate (Table 6.8).

V. Mating Reactions Among the Interspecific Heterokaryons and the Testers.

Interspecific heterokaryons of SF1 were crossed with the four P. sajor-caju testers including S22, S23, S27 and S36, and the P. florida testers F1, F6, F12, and F13. In the previous study (chapter 5), Su223 and Fu156 which were the parential strains of SF1 could result in positive mating with S23 and F6, respectively. Microscopic observation revealed that only SF1-2 and SF1-5 failed to form clamp connections with S23. It was noted that the successful dikaryotization was restricted to the mycelia of the monokaryotic testers. However, all SF1 heterokaryons did not undergo dikaryotization with F6 (Table 6.9). The mating reactions of SF1-9 with the parental testers were shown in Fig. 6.7. The result could be reproducible after three - month culture of heterokaryons. Moreover, the dikaryotized mycelia could form fruit bodies which were similar to that of P. sajor-caju in morphology, but much smaller in size (Fig. 6.8). In contrast, SF2 and SF3 heterokaryons could form clamp connections with F6, one of the P. florida testers, but not with any testers of P. sajor-caju (Table 6.10 and 6.11). The mating reactions of SF2 and SF3 with the parental

Table 6.8 Number of nuclei in apical cells and subapical cells of isolates of interspecific heterokaryon (SF2) of Pleurotus.

Isolate	Apical cell		Subapical cell	
	2	3-5	2	3-5
SF2-1	17	0	41	2
SF2-2	22	1	50	4
SF2-3	15	0	33	3
SF2-4	28	5	46	1
SF2-5	31	3	49	5

Table 6.9 Mating reactions among isolates of interspecific heterokaryon (SF1) of Pleurotus with monokaryotic testers from P. sajor-caju and P. florida (+, dikaryotization; -, no dikaryotization.)

Isolate	Tester							
	P. sajor-caju				P. florida			
	S22	S23	S27	S36	F1	F6	F12	F13
SF1-1	-	+	-	-	-	-	-	-
SF1-2	-	-	-	-	-	-	-	-
SF1-3	-	+	-	-	-	-	-	-
SF1-4	-	+	-	-	-	-	-	-
SF1-5	-	-	-	-	-	-	-	-
SF1-6	-	+	-	-	-	-	-	-
SF1-7	-	+	-	-	-	-	-	-
SF1-8	-	+	-	-	-	-	-	-
SF1-9	-	+	-	-	-	-	-	-
SF1-10	-	+	-	-	-	-	-	-
SF1-11	-	+	-	-	-	-	-	-

Fig. 6.7 Mating reaction among the pairings between monokaryotic testers from Pleurotus sajor-caju (left) and from P. florida (right) and interspecific heterokaryon, SF1-9.

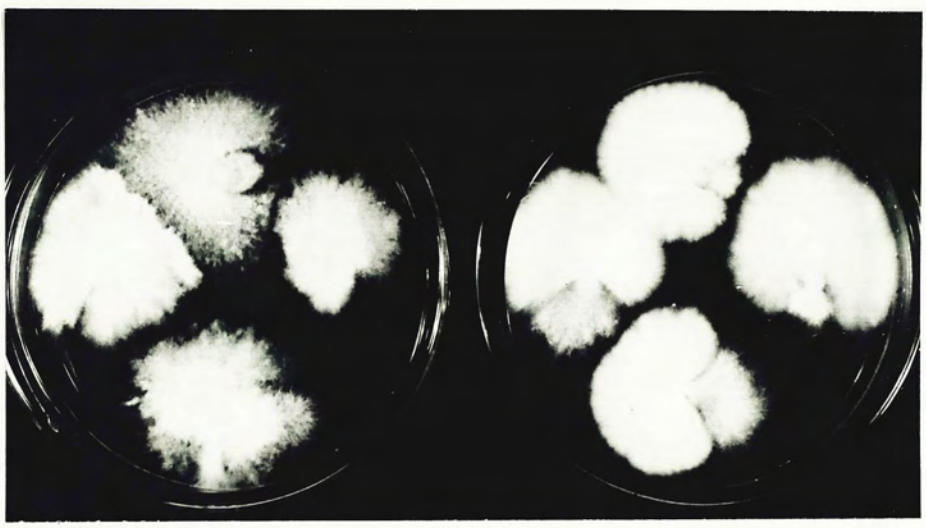


Fig. 6.8 Fruit body formation (indicated by arrows) on dikaryons resulted from dikaryotilization of monokaryotic strain of Pleurotus sajor-caju (S23) by interspecific heterokaryon, SF1.

From left to right: SF1-6 x S23, SF1-7 x S23, SF1-8 x S23, SF1-9 x S23 and SF1-10 x S23.

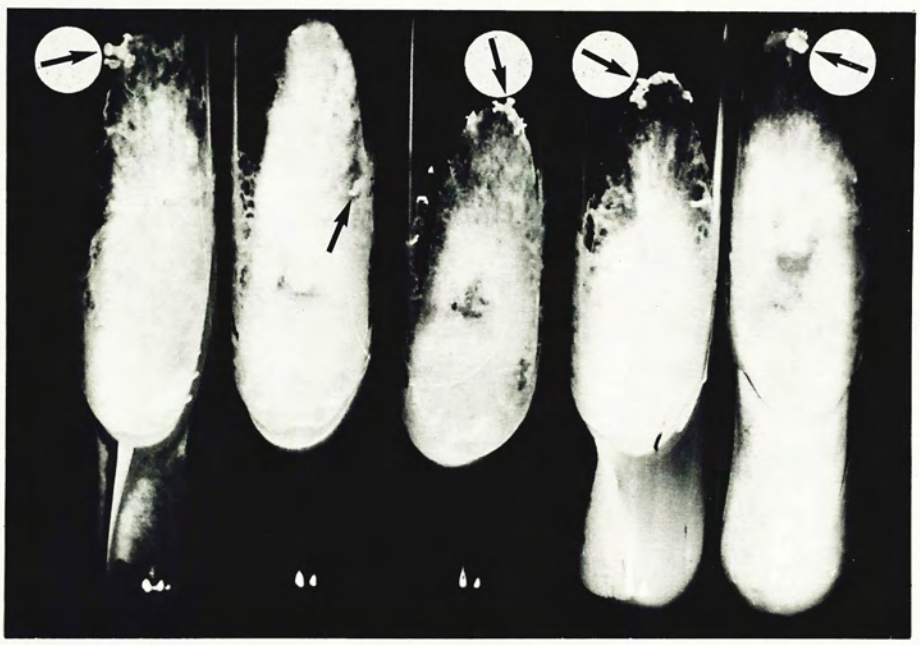


Table 6.10 Mating reactions among isolates of interspecific heterokaryon (SF2) of Pleurotus with monokaryotic testers from P. sajor-caju and P. florida (+, dikaryotization; -, no dikaryotization.)

Isolate	Tester							
	P. sajor-caju				P. florida			
	S22	S23	S27	S36	F1	F6	F12	F13
SF2-1	-	-	-	-	-	+	-	-
SF2-2	-	-	-	-	-	+	-	-
SF2-3	-	-	-	-	-	+	-	-
SF2-4	-	-	-	-	-	+	-	-
SF2-5	-	-	-	-	-	+	-	-

Table 6.11 Mating reaction among isolates of interspecific heterokaryon (SF3) of *Pleurotus* with monokaryotic testers from *P. sajor-caju* and *P. florida* (+, dikaryotization; -, no dikaryotization.)

Isolate	Tester							
	P. sajor-caju				*P. florida*			
	S22	S23	S27	S36	F1	F6	F12	F13
SF3-1	-	-	-	-	-	+	-	-
SF3-2	-	-	-	-	-	+	-	-
SF3-3	-	-	-	-	-	+	-	-
SF3-7	-	-	-	-	-	-	-	-
SF3-8	-	-	-	-	-	+	-	-

testers was shown in Figs. 6.9, a and b.

VI. Comparison of Zymograms of Alcohol Dehydrogenase (ADH) and Esterase

In a preliminary experiment, 4 isoenzymes, including peroxidase, malate dehydrogenase, ADH and esterase were tested in 4 auxotrophic mutants used for protoplast fusion. A mixture of extracts from the two parental strains served as a control. Peroxidase yielded a multiplicity of bands which were similar that in 4 strains, and thus it was unsuitable for use to distinguish the strains. For malate dehydrogenase, a single band existed in the auxotrophic mutants of P. major-caju, but no such clear band(s) could be observed in the mutants of P. florida. In contrast, ADH and esterase had distinctive bands distribution in the mutants of P. major-caju and P. florida (Figure 6.10). The staining time for esterase isoenzyme bands required 5-10 min , but ADH required 30 min. Moreover, the band patterns of these isoenzymes were very stable and showed no difference among 2-week, 4- week and 6-week mycelial cultures. The Ful56 mutant gave two major and fast bands of esterase at Rf 0.85 and 0.94 and a slow band at 0.4. The auxotroph Su223 consistantly possessed major bands of esterase at Rf 0.25, 0.383 and 0.633. On the other hand, Ful56 had a slower band of ADH

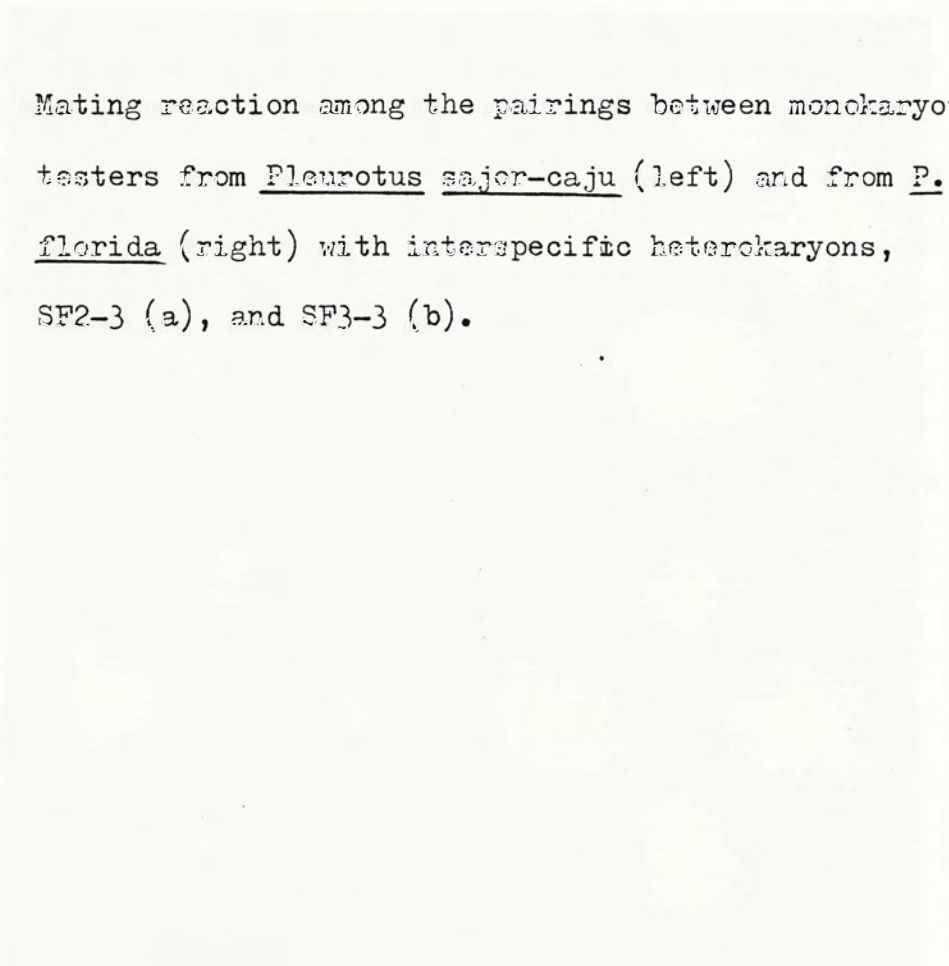


Fig. 6.9 Mating reaction among the pairings between monokaryotic testers from Pleurotus sajor-caju (left) and from P. florida (right) with interspecific heterokaryons, SF2-3 (a), and SF3-3 (b).

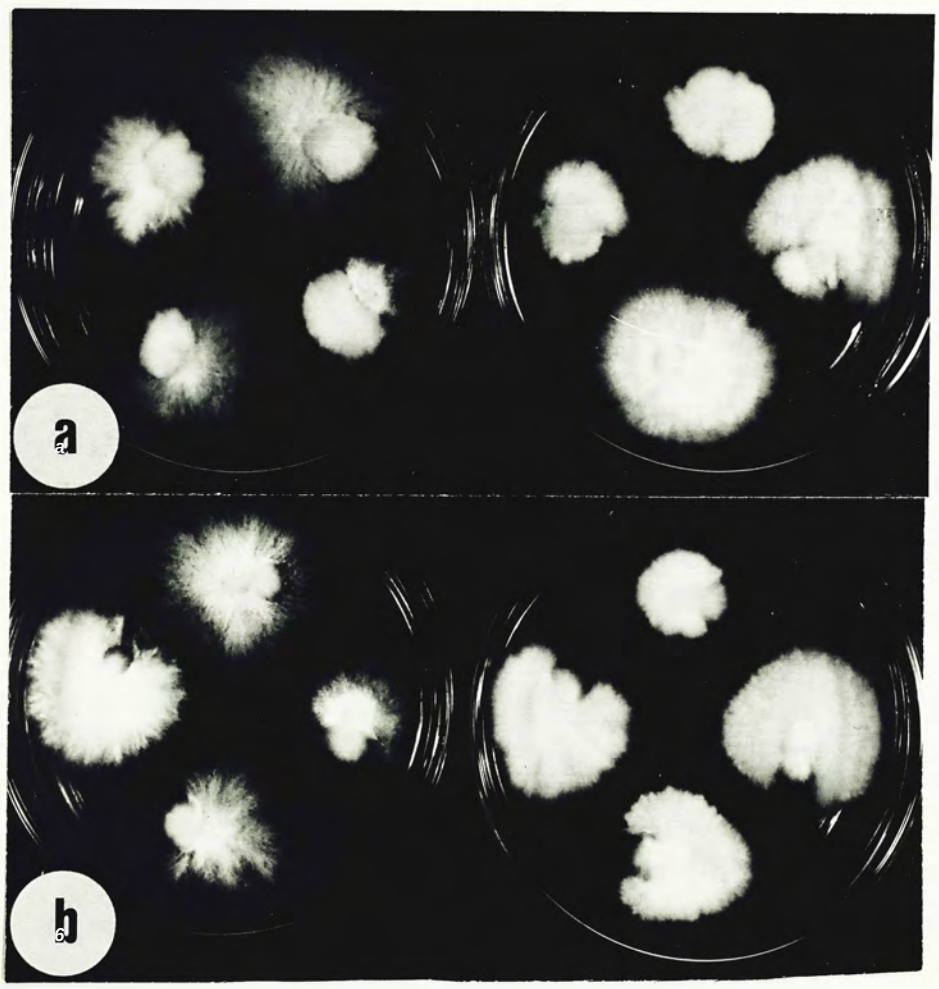
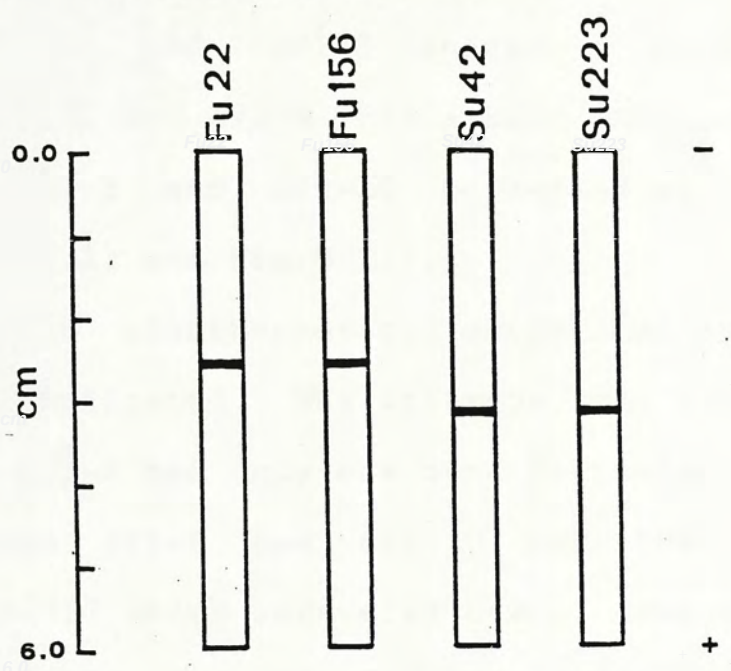
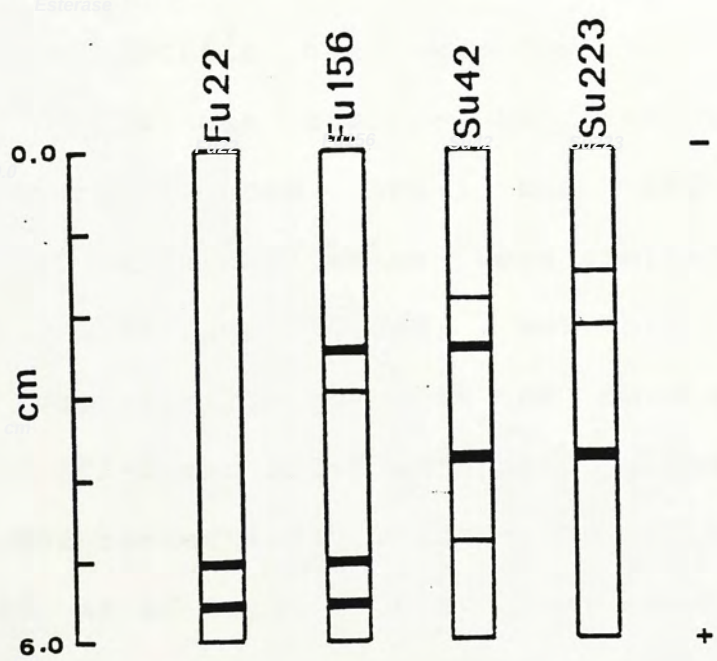

a
b

Fig. 6.10 Zymograms of alcohol dehydrogenase (a) and esterase (b) from auxotrophs of Pleurotus sajor-caju (Su42 and Su223) and of P. florida (Fu22 and Fu156).

a/ Alcohol dehydrogenase



b/ Esterase



at Rf of 0.433 and Su223 had a fast band at Rf 0.533. The composite electrophoretic pattern of ADH showed that the interspecific heterokaryons of SF1-4 and SF1-5 shared a common band as that of Fu156, and SF1-6 had a band similar to that of Su223. Only SF1-1 and SF1-10 possessed an intermediate band (Table 6.12 and Fig.6.11).

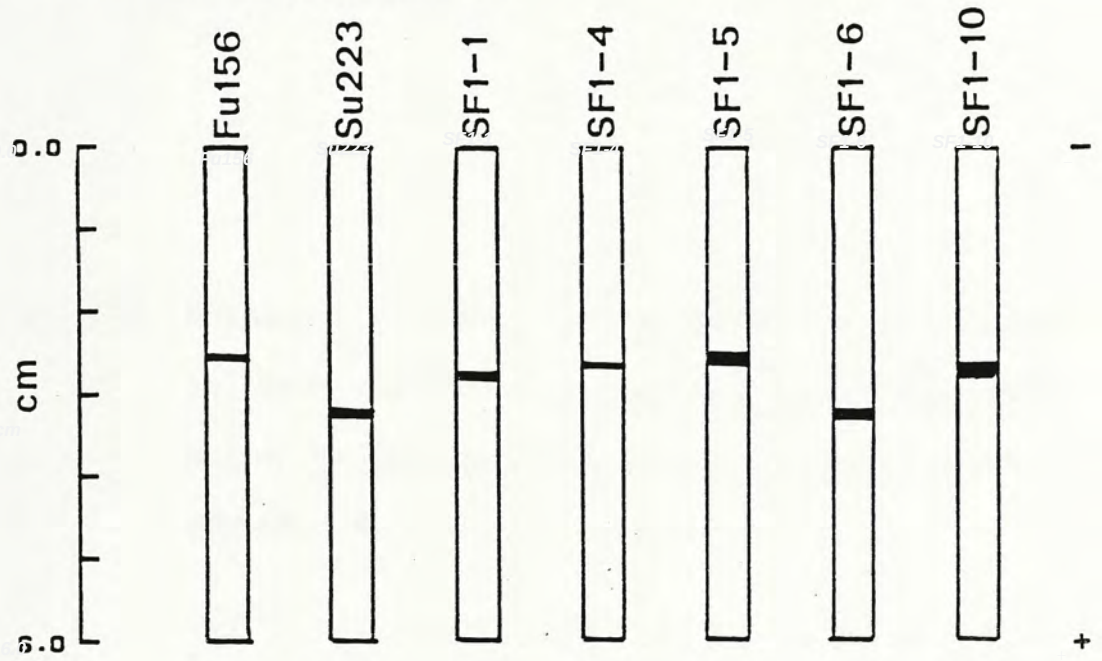
The electrophoretic pattern of esterase in SF1 was more complicated. The interspecific heterokaryons of SF1-1 and SF1-5 had only one band for esterase which was novel and both SF1-6 and SF1-10 had the bands at Rf 0.3 and 0.717 which were also new . However, SF1-6 had two more bands at Rf 0.4 and 0.442. SF1-4 had two bands which were similar to that of Su223 at Rf 0.25 and 0.383 and one band at Rf 0.683 (Table 6.12). In the case of SF2, the electrophoretic patterns showed that all the interspecific heterokaryons had one common band of ADH which was similar to that of Su42 (Fig. 6.12) In contrast, both SF2-1 and SF2-2 had 2 common bands of esterase which were similar to that of Fu22 at Rf 0.85 and 0.942, but had another one band which was similar to that of Su42 at Rf 0.333 (Table 6.13). SF2-3 and SF2-5 each had one fast band at Rf 0.867 and 0.892 respectively which seemed to be the same one as in Fu22 at Rf 0.85. Other fast bands at Rf 0.767 and 0.792 were quite similar to that at Rf 0.767 in

Table 6.12 R_f value on zymograms of alcohol dehydrogenase and esterase from isolates of interspecific heterokaryon (SF1) of Pleurotus.

Isolate	Alcohol dehydrogenase	Esterase
Fu156	0.433	0.40, 0.850, 0.942
Su223	0.533	0.250, 0.383. 0.633
SF1-1	0.483	0.733
SF1-4	0.467	0.250, 0.383, 0.683
SF1-5	0.467	0.683
SF1-6	0.533	0.30, 0.40, 0.442, 0.717
SF1-10	0.483	0.30, 0.717

Fig. 6.11 Zymograms of alcohol dehydrogenase (a) and esterase (b) from auxotrophs of Pleurotus sajor-caju (Su223) and of P. florida (Fu156), and isolates of inter-specific heterokaryon (SF1).

a/ Alcohol dehydrogenase



b/ Esterase

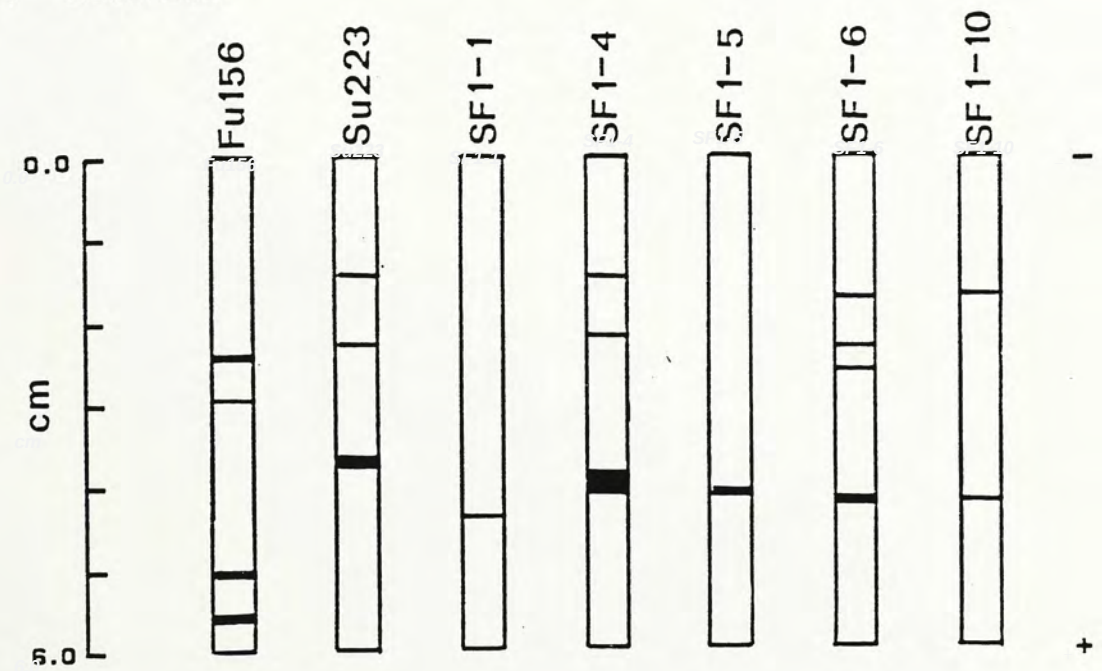
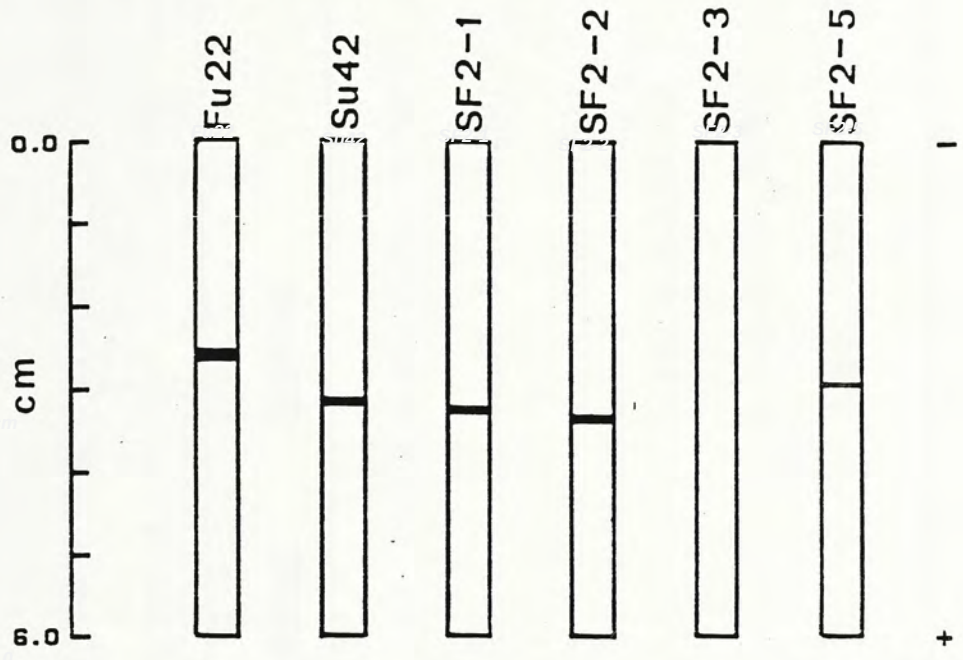


Fig. 6.12 Zymograms of alcohol dehydrogenase (a) and esterase (b) from auxotrophs of Pleurotus sajor-caju (Su42) and of P. florida (Fu22), and isolates of inter-specific heterokaryon (SF2).

a/ Alcohol dehydrogenase



b/ Esterase

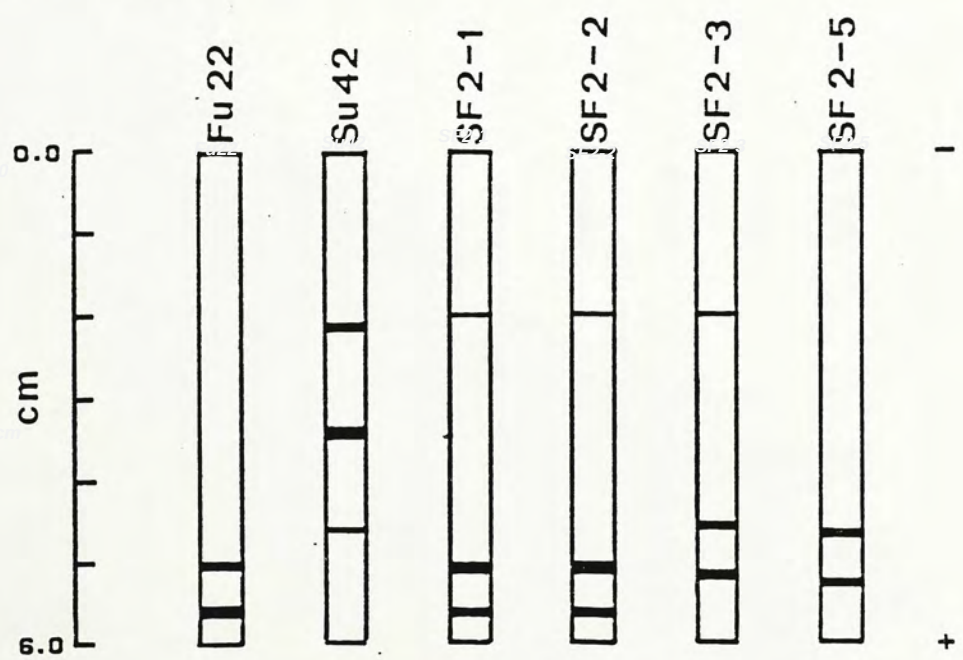


Table 6.13 R_f value on zymograms of alcohol dehydrogenase and esterase from isolates of interspecific heterokaryon (SF2) of Pleurotus.

Isolate	Alcohol dehydrogenase	Esterase
Fu22	0.433	0.40, 0.85, 0.942
Su42	0.533	0.367, 0.583, 0.767
SF2-1	0.55	0.333, 0.850, 0.942
SF2-2	0.567	0.333, 0.850, 0.942
SF2-3	-*	0.333, 0.767, 0.867
SF2-5	0.50	0.792, 0.892

*Not tested.

Su42.

C. DISCUSSION AND CONCLUSION

I. Fusion Frequency

The introduction of PEG is an important improvement in protoplast fusion. Generally speaking, PEG of MW 4000 to 6000 at 30% concentration is effective in many fungi including Penicillium (Anne and Peberdy, 1975), Aspergillus (Ferenczy et al., 1977) and Mucor (Ohnuki et al., 1982). When observed under the phase contrast microscope, fusion among Pleurotus protoplasts could be detected after 10 min treatment with 30% PEG. The fusion frequency which was determined by the ratio of mycelial colonies grown on the minimal medium and on the complete medium ranged from 2.6 - 12.08% in three different fusion experiments. This complementation frequency could be compared to that of other fungal species.

The frequency of heterokaryon formation in interspecific hybridization between Aspergillus nidulans + A. rugulosus was from 2.23 % - 6.99 % and intraspecific fusion frequency was about 13% (Kevei and Peberdy, 1977). The fusion frequency obtained in above instance was higher than that in intraspecific fusion in various Aspergillus and Penicillium as reported by Anne and Peberdy (1976). They

used a washing procedure after PEG treatment of the protoplast mixture. Kevei and Peberdy (1977) suggested washing could break up the protoplst aggregates which tended to fuse together as shown by colony development on the minimal medium , but increase the colony number on the complete medium. The fusion frequency in P. chrysogenum + P. cyaneofulvum crosses ranged from 0.09 - 0.2% (Peberdy et al., 1977), and 0.03 - 3.4 % in P. roqueforti + P. chrysogenum (Anne et al., 1976). On the other hand , 5 - 30% fusion frequency was obtained in the interspecific hybridization between Mucor pusillus + M. meihei (Ohnuki et al., 1982). For yeast, the complementation frequency was about 0.6% in the intraspecific fusion between Saccharomyces cerevisiae (Ferenczy and Maraz , 1977), and 0.17% in Schizosaccaromyces pombe (Sipiczki and Ferenczy, 1977). However, the fusion frequency of 3×10^{-5} was very low in Candida tropicalis (Fournier et al., 1977). The fusion frequency in interspecific hybridization was $10^{-4} - 10^{-3}$ between Schizosaccharomyces pombe + S. octosporus (Sipiczki , 1979). It was suggested that significant differences did occur between the genomes of two partners which were taxonomically closed related. After the protoplast fusion, such differences could prove lethal or result in anomalous growth followed by a very low ciability in the fusion products. The hybrid cells could be classified

into two main morphological types: type 1 with irregularly spheroidal form which differed from both parents and type 2 showed S. octosporus morphology. However, the fusion products were greatly unstable and segregated immediately into auxotrophic cells in the absence of nutritional pressure. As the instances in Penicillium (Peberdy et al., 1977) and Aspergillus (Ferenczy et al., 1977), only one parent, S. octosporus, could be re-isolated. In contrast, the interspecific hybrids in Kluyveromyces lactis + K. fragilis maintained their prototrophy after prolonged growth on complete medium (Whittaker and Leach., 1978). Moreover, the hybrids obtained from the fusion between Saccharomyces montanus + S. diastaticus, S. rosei + S. cerevisiae (P-), Candida pseudotropicalis + S. cerevisias (P-), Pichia membranefaciens + S. cerevisiae (P-) appeared to be relatively stable on the selective medium. No morphological variants were observed when colonies were plated on other media (Spencer and Spencer, 1981).

In filamentous fungi, the interspecific heterokaryons of Aspergillus nidulans + A. fumicatus were unstable on yeast extract medium and produced many rapidly growing sectors. Neither inoculation of mycelia or conidia formed on these sectors could grow on minimal medium (Ferenczy et al., 1977). The parental strains were readily segregated when heterokaryons of A. nidulans + A. rugulosus

grew on complete medium (Kevei and Peberdy, 1977). In the case of Penicillium chrysogenum + P. cyaneo-fulvum, the heterokaryons were stable and showed no visible evidence of sectoring. However, this stability could be broken down and prototrophic and auxotrophic colonies were isolated when the heterokaryons were cultivated on complete medium supplemented with P- fluorophenylalanine or benomyl (Peberdy et al., 1977).

The colonies of the interspecific heterokaryons did not show any morphological difference in the fusion between Su223 + Ful56, and also in the case of Su42 + Fu22. Two distinctive mycelial colony types were observed among the heterokaryons from Su42 + Ful56. All these interspecific heterokaryons had to be the result from protoplast fusion between two different parental strains, e.g. Su223 + Ful56. However, no rapid-growing sectors could be observed when these heterokaryons, isolated from three different fusion events, were grown on complete medium.

II. Sexual Backcrosses of Interspecific Heterokaryons

Sexual backcrosses of the fusion products to the parental testers were carried out for two main purposes: (1) to testify whether or not the isolates grown on minimal medium were true fusion products or just contaminants; and (2) to study the mating reactions with

both parental testers for investigation on the incompatibility systems between P. florida and P. sajor-caju.

From a cytological study on nuclear staining, there appeared that many old mycelial cells which nuclei could not be stained. It might be due to the reason that some unknown material, secreted from the cells, protected the cell wall surface and thus blocking the entrance of the dye. Therefore, only the nuclear number in the apical cells and the subapical cells, which consisted of only 3-4 cells, were determined .

It could be concluded that cells of the isolated interspecific heterokaryons were generally binucleate Theoretically, the two nuclei in each heterokaryotic cells could be contributed by both parental fusion partners for fulfilling the outcome of nutrient complementation. By pairing with the four testers of each parents, the interspecific heterokaryons might at least give positive reaction with one of the testers of each parent respectively. These matings were quite similar to the di-mon matings in Buller Phenomenon (Raper, 1966). When the nucleus in the homokaryon is compatible with neither type of the dikaryon the mating is noncompatible and illegitimate, e.g., (A1B2 + A2B1) + A1B1. In incompatible matings, both nuclei of the dikaryon may eventually migrate through the monokaryotic hyphae and establish the original genome dikaryon within the hyphal tips occasionally. In

hemicompatible di-mon matings, e.g., (A1B1 + A2B2) + A1B1, nuclear selection operate in favour of the compatible A- and B - factors. In a fully compatible di-mon mating, e.g., (A1B1 + A2B2) + A3B3, re-establishment of the di-karyon within the tips of the monokaryons could occur.

However, nonrandom or preferential selection of the two nuclear components in the original dikaryons often occurred in the establishment of derived dikaryon from the fully compatible di-mon matings. In an experiment using a large number of compatible di-mon matings of Coprinus macrorhizus, Kimura (1958) observed a strong preferential selection of nonsibling nuclei over sibling nuclei by the homokaryotic mates in the dikaryotization of the homokaryons. Such phenomenon was also demonstrated in Schizophyllum commune (Ellingboe and Raper, 1962). According to these results, the selective system operated in compatible di-mon matings appeared to favour the association of nuclei which have the greater degree of genetic heterogeneity. By a detailed study, it was found that nuclei of both dikaryotic components entered the homokaryons and they seemed to migrate and multiply at the same rate. It was concluded that the preferential selection only reflected earlier arrival of one type nucleus at the growing hyphal tips of the monokaryons (Ellingboe, 1964).

Furthermore, matings between monokaryons and

dikaryons of three P. ostreatus isolates including Florida strain (F), multispore culture (M), and west Germany strain (G) have been studied in detail by Eger and coworkers (1976). In the pairings of F-mon with M-di and vice versa, dikaryotization of the monokaryons could proceed regularly and rapidly. However, dikaryotization took place in only a fraction of the matings with one partner of German origin (e.g. G-mon X F-di, F-mon X G-di). They suggested that these results actually reflected the differences in nuclear and plasmatic interactions. In such a complicated process of di-mon mating, the conjugated nuclear division has to require cooperation not only between the gene(s) at the sexual incompatibility loci, but also possibly other gene(s) as well, and with the cytoplasmic factor(s).

In the hetero-mon matings between interspecific heterokaryons SF1 and monokaryotic testers of P. sajor - caju, it was found that the majority of heterokaryons could cross positively with S23. However, dikaryotization in matings between SF1-heterokaryons and the four monokaryotic testers of P. florida was not observed. The failure could be due to many unknown factors such as nuclear-cytoplasmic interaction, and the deficiency of incompatibility factors by the chromosome elimination of parental strain (Ful56) after protoplast fusion. According to Ellingboe's hypothesis, the result could also be

due to the earlier arrival of Su223 nucleus at the hyphal tips of monokaryon, thus interfered with the formation of clamp connection by the interaction between the nuclei of P. florida, e.g. the Ful56 and F6.

On the other hand, in heterokaryons, the two nuclei coming from different parents could not compatibly interact with any one monokaryotic testers of P. florida. This result implied that the incompatibility systems in P. sajor-caju and P. florida were distinctively different. In the mating experiment, monokaryon-pairings between "Florida + German strains ", "multiple spore culture + Florida strain ", or " German strain + multiple spore culture " were 100% compatible and showed that A- and B- factors were different in multiple alleles at two incompatibility factor's loci (Eger et al., 1976). Moreover, from the study of interstock pairings among the four mating-type isolates of 12 different P. ostreatus sporophores obtained from three locations in America, it was found that monokaryotic testers of seven among them were 100% compatible with all other testers except the isolates of one sporophore. Based on this result, it was estimated that there were approximately 63 A factors and a minimum of 180 B factors in P. ostreatus population (Eugenio and Anderson, 1968). In contrast, interspecific heterokaryons of SF2 and SF3 could only form dikaryotization with F6 which was one of the four testers of P. florida

but not with any tester of P. sajor-caju. It was noted that both SF2 and SF3 used Su42 as one parental strain which was different from SF1 to hybridize with Fu22 and Ful56 , respectively. It appeared that preferential nuclear selection of Fu22 had occurred in SF2 and of Ful56 in SF3.

III. Fructification Tests of Interspecific Heterokaryons

However, isolates of interspecific heterokaryons of Pleurotus did not form fruit bodies. Among them, SF1-1, SF1-5, SF1-6 and SF1-7 could produce primordia, but these did not develop further. In Coprinus macrorhizus several mutants, such as initiationless, primordiumless, maturationless, elongationless, and expansionless, which interfered with the fruiting at corresponding stages, were obtained and these mutants were found to be dominant to the normal gene in dikaryons (Takemaru and Kamada, 1971). Therefore, it was suggested that the fruiting in Pleurotus was also controlled by a number of genes operating at different stages of development (Eger et al., 1976). Stahl and Esser (1976) also suggested that the development of fruit bodies in Polyporus ciliatus was strictly under genetic control. In this species, the fi gene controlled the formation of dikaryotic fruit bodies in two aspects: the allele fi+

could enhance fruit body development whereas the allele fi may inhibit fruit body development completely. The two other genes only determine the shape of the fruit bodies (fb+/fb) and its fertility (mod+/mod). They also concluded that the complex incompatibility factors controlled the whole sequence of morphogenesis from plasmogamy to karyogamy and meiosis in Basidiomycetes. However, fi dikaryons could not fruit at all even though they had the constitution of A*B* in the most extreme case. Furthermore, strains of Agrocybe aegerita having the fi+/fb genotype developed only spherical fruiting initials and fruiting did not occur in the presence of the inactive allele, fi,e.g., fi/fb or fi/fb+ genotype (Esser and Meinhardt,1977; Meinhardt and Esser, 1981). The elucidation of the genetic control of fructification could imply that the development of fruit body in interspecific heterokaryons may encounter many difficulties.

IV. Comparison of Isoenzyme

In auxotrophic mutants, Su42, Su223, Fu22 and Ful56, only one ADH band was detected in the mycelia. ADH could be found in many plant species (Scandalios, 1974) and edible mushroom (Royse and May, 1982). Kut and Evans (1984) reported that in Nicotiana, among 20 species tested, 16 had only one band of ADH. All sexual and

somatic hybrids which were obtained from N. tabacum x N. glauca, each had only one band, had both parental bands. Study on isoenzyme patterns in soybean-Nicotiana somatic hybrid cell lines showed that soybean and N. glauca only had a single band of ADH isoenzyme with different Rf whereas all the hybrid lines had the double band patterns indicating that one was originated from N. glauca and the other from soybean. However, the band derived from Nicotiana has been lost after 8 months of culture. Such changes in electrophoretic zymograms of the hybrids after a long period of culture appeared to be associated with chromosomal loss (Watter, 1977). Moreover, zymograms revealed that Arabidopsis thaliana had 3 bands and Brassica campestris had four bands of ADH. Both parents had two identical major bands, and only these 2 bands could be detected in the somatic fusion hybrids of these two species (Gleba and Hoffmann, 1978).

Absence of one parental ADH band from the zymograms of both interspecific heterokaryons SF1 and SF2, could be due to the chromosome loss or the enzyme activity was too low to be detected by colour staining. Zymogram of esterase was more complicated than that of ADH in the four auxotrophs and the interspecific heterokaryons. Similar observations were also found in many other reports. Different hybrids lines had different comparative intensities of esterase bands but 2 - 3 parental bands often disappeared in the

hybrids (Gleba and Hoffmann, 1978).

Electrophoresis of esterase isoenzyme banding patterns of F1 pea lines obtained from sexual crosses also showed variation although the majority of them contained bands from both parental lines (Mahmond et al, 1984). Furthermore, the comparison of interspecific hybrids between Penicillium chrysogenum + P. roqueforti using isoenzyme analysis showed that the hybrids and their segregants contained enzymes either similar to P. chrysogenum, or to P. roqueforti or to both parents. These results could demonstrate that the interaction of the two genomes existed in the interspecific hybrids (Anne and Peberdy, 1981).

From the analysis of isoenzyme banding patterns of ADH and esterase in the interspecific heterokaryon between P. sajor-caju + P. florida, it might be concluded that the genomes of both species did exist in the heterokaryons and their differential interaction gave the various expression of the genotype.

CHAPTER 7

GENERAL CONCLUSION

From the experiments of mating tests and cytological study, it could be suggested from the following observation that there was no hyphal fusion between Pleurotus sajor-caju and P. florida:

(1) There was always a formation of contact zone between the pairing monokaryotic inocula but there was no formation of clamp connection as observed in any pairing.

(2) The contact zone have repeatedly reappeared in each subsequently subculture of inocula from the original contact zone.

(3) Only one nucleus per cell was observed in the mycelia at the margin of above subculture.

The above results were also observed in the mating of mutants of these two species.

In this case, protoplast fusion could provide the opportunity for the genetic exchange and recombination between these two Pleurotus species. In the several control experiments set up for the protoplast fusion, the back-mutation could not be obtained among the protoplast population. Thus, the colonies resulting from protoplast reversion on minimal medium should have been the outcome

of nutrient complementation of two parental nuclei in the same cell through protoplast fusion. Further evidence from the number of nuclei per cell and the banding patterns of isoenzymes, also confirmed that these isolates were interspecific heterokaryons. Although each interspecific heterokaryotic isolate could dikaryotize the monokaryotic testers of only one species, either P. sajorcaju or P. florida, it could at least be concluded that isolated heterokaryons obtained from PEG - treated protoplasts were not contaminated, and more importantly, were not a mycelial mixture of both parents.

Confirmation of the protoplast fusion hybrid nature requires a demonstration of genetic contributions from both parents. The expression from both parents in the form of identifiable biochemical markers could provide the most convincing and useful evidence for these interspecific hybrids. However, considerable differences in expression (or repression) do occur among individual hybrid lines from the same two parental species or strains and even between different plants derived from a single hybrid cell line. Interspecific protoplast fusion does not necessarily yield population of somatic hybrid with a uniform phenotype or the expression of designated molecular markers. It has been suggested that both nuclear and extranuclear gene(s) expression might contribute to such differences because protoplast fusion

produced hybrid cells that contained mixed organelles as well as mixed nuclear components (Shepard et al., 1983). Anne, in his review in 1983, stated that protoplast fusion using nutritionally complementary strains, could form different types of prototrophs which reflected the variation observed in the isoenzyme banding patterns. Moreover, the different expression of somatic hybrids was probably the consequence of selective loss of chromosome (Anne, 1983; and Peberdy et al., 1981).

The repeated attempts to obtain fruit bodies from interspecific heterokaryons were unsuccessful. The failure of fructification could have been the result of many genetic problems.

Somatic hybrids themselves are usually not of immediate value in plant breeding, especially in the interspecific crosses between sexually incompatible species. But with subsequent back-crossing, by which undesirable characters could be eliminated to improve the strains as is in the case of conventional sexual breeding, such novel somatic hybrids are valuable as starting materials for a genetic introgression scheme.

REFERENCE

Ahkong, Q. F., Fisher, D., Tampion, W, and Lucy, J.A. 1975. Mechanisms of cell fusion. Nature (London) 253,194-195.

Allmark, B.M., Morgan, A.J., and Whittaker, P.A. 1978. The use of protoplast fusion in demonstrating chromosomal and mitochondrial inheritance of respiratory-deficiency in Kluyveromyces lactis, a petite-negative yeast. Mol. Gen. Genet. 159, 297-299.

Anderson, F.B., and Millbank, J.W. 1966. Protoplast formation and yeast cell-wall structure. Biochem. J. 99, 682-687.

Anderson, N.A., Wang, S.S., and Schwandt, J.W. 1973. The Pleurotus ostreatus-sapidus species complex. Mycologia 65, 28-35.

Anne, J. 1983. Protoplasts of filamentous fungi in genetics and metabolite production. In: Protoplasts, 1983 Lecture Proceedings 6th International Protoplast Symposium. pp. 167-178. Edited by Potrykus, I., Harms, C.T., Hinnen, A., King, P.J., and Shillito, R.D. Birkhauser Verlag, Boston.

Anne, J., Eyssen, H., and de Somer, P. 1974. Formation and regeneration of Penicillium chrysogenum protoplasts. Arch. Microbiol. 98, 159-166.

Anne, J., Eyssen H., and de Somer, P. 1976. Somatic hybridization of Penicillium roqueforti with P. chrysogenum

after protoplast fusion. Nature (London) 262, 719-721.

Anne, J., Peberdy, J.F. 1975. Conditions for induced fusion of fungal protoplasts in polyethylene glycol solutions. Arch. Microbiol. 105, 201-205.

Anne, J., and Peberdy, J.F. 1976. Induced fusion of fungal protoplasts following treatment with polyethylene glycol. J. Gen. Microbiol. 92, 413-417.

Anne, J., and Peberdy, J. F. 1981. Characterization of interspecific hybrids between Penicillium chrysogenum and P. roqueforti by iso-enzyme analysis. Trans. Br. Mycol. Soc. 77, 401-408.

Bachmann, B.J., and Bonner, D.M. 1959. Protoplasts from Neurospora crassa. J. Bacteriol. 78, 550-556.

Ben-Hayyim, G., Shani, A., and Vardi, A. 1982. Evaluation of isozyme systems in Citrus to facilitate identification of fusion products. Theor. Appl. Genet. 64, 1-5.

Binding, H. 1966. Regeneration and fusion behavior of isolated protoplasts of mosses. Z. Pflanzenphysiol. 55, 305-321.

Binding, H., Jain, S.M., Finger, J., Mordhorst, G., Nehls, R., and Gressel, J. 1982. Somatic hybridization of an atrazine resistant biotype of Solanum nigrum with Solanum tuberosum. Theor. Appl. Genet. 63, 273-277.

Binding, H., and Weber, H.J. 1974. The isolation, regeneration and fusion of Phycomyces protoplasts. Mol. Gen. Genet. 135, 273-276.

Blaschek, W., Hess, D., and Hoffmann, F. 1974. Transcription in

nuclei prepared from isolated protoplasts of Nicotiana and Petunia. Z. Pflanzenphysiol. 72, 262-271.

Block, S. S., Tsao, G., and Han, L. 1958. Production of mushrooms from sawdust. J. Agric. Food Chem. 6, 923-927.

Block, S.S., Tsao, G., and Han, L. 1959. Experiments in the cultivation of Pleurotus ostreatus. Mush. Sci. 4, 309-325.

Boisselier-Dubayle, H.C. 1983. Taxonomic significance of enzyme polymorphism among isolates of Pleurotus (Basidiomycetes) from Umbellizers. Trans. Br. Mycol. Soc. 81, 121-127.

Bonnett, H. T., and Erikson, T. 1974. Transfer of algal chloroplasts into protoplasts of higher plants. Planta 120, 71-79.

Bos, C.J., and Slakhorst, S.M. 1981. Isolation of protoplasts from Aspergillus nidulans conidiospores. Can. J. Microbiol. 27, 400-407.

Boyce, R.P., and Howard-Flanders, P. 1964. Release of ultraviolet light-induced thymine dimers from DNA in E. coli K-12. Proc. Natl. Acad. Sci. USA 51, 293-300.

Brown, J.P. 1971. Susceptibility of the cell walls of some yeasts to lysis by enzymes of Helix pomatia. Can. J. Microbiol. 17, 205-208.

Burgess, J., and Fleming, E.N. 1974. Ultrastructural studies of the aggregation and fusion of plant protoplasts. Planta 118, 183-193.

Burgess, J., and Linstead, P.J. 1976. Scanning electron microscopy of cell wall formation around isolated plant

protoplasts. Planta 131, 173-178.

Burnett, J.H. 1975. Mycogenetics. John Wiley and Sons, London.

Button, J., Vardi, A., and Spiegel-Roy, P. 1976. Root peroxidase isoenzymes in Citrus breeding. Theor. Appl. Genet. 47, 119-123.

Carlson, P.S. 1973. The use of protoplasts for genetic research. Proc. Nat. Acad. Sci. USA 70, 598-602.

Chang, S.T., Lau, O.W., and Cho, K.Y. 1981. The cultivation and nutritional value of Pleurotus sajor-caju. Appl. Microbiol. Biotechnol. 12, 58-62.

Chu, S. B., and Alexander, M. 1972. Resistance and susceptibility of fungal spores to lysis. Trans. Br. Mycol. Soc. 58, 489-497.

Cocking, E.C. 1960. A method for isolation of plant protoplasts and vacuoles. Nature (London) 187, 962-963.

Cocking, E. C. 1966. An electron microscopic study of the initial stages of infection of isolated tomato fruit protoplasts by tobacco mosaic virus. Planta 68, 206-214.

Cocking, E. C. 1972. Plant cell protoplasts - isolation and development. Ann. Rev. Plant Physiol. 23, 29-50.

Cocking, E.C., Davey, M.R., Pental, D., and Power, J.B. 1981. Aspects of plant genetic manipulation. Nature (London) 293, 265-271.

Cook, K.A. 1974. Regulation of aromatic metabolism in fungi: Selection of mutants of the yeast Rhodotorula mucilaginosa with nystatin. J. Gen. Microbiol. 85, 29-36.

Cove, D.J. 1979. The uses of isolated protoplasts in plant genetics. Heredity 43, 295-314.

Crespi, H.L., Mandeville, S.E., and Katz, J.J. 1962. The action of lysozyme on several blue-green algae. Biochem. Biophys. Res. Commun. 9, 569-573.

Croft, H. J. and Dales, R.B. 1979. Protoplast fusion and vegetative incompatibility in Aspergillus nidulans. In.: Protoplasts - Applications in Microbial Genetics. pp. 27-34. Edited by Peberdy, J.F. University of Nottingham, England.

Croft, H.J., and Dales, R.B. 1983. Interspecific somatic hybridization in Aspergillus. In: Protoplast, Lecture Proceedings 6th International Protoplast Symposium. pp. 179-186. Edited by Protrykus, I., Harms, C.T., Hinnen, A., King, P.J., and Shillito, R.D. Birkhauser Verlay, Boston.

Davey, M.R., and Cocking, E.C. 1972. Uptake of bacteria by isolated higher plant protoplasts. Nature (London) 239, 455-456.

Davies, R. and Elvin, P.A. 1964. The effect of B - mercaptoethanol on release of invertase and formation of protoplasts of Saccharomyces fragilis. Biochem. J. 93, 8.

Davis, B.J. 1964. Disc electrophoresis. II. Method and application to human serum proteins. Ann. N. Y. Acad. Sci. 121, 404-427.

De Serres, F.J., and Kolmark, H.G. 1958. A direct method for determination of forward- mutation rates in Neurospora

crassa. Nature (London) 184, 1249-1250.

de Vries, O.M.H., and Wessels, J.G.H. 1972. Release of Protoplasts from Schizophyllum commune by a lytic enzyme preparation from Trichoderma viride. J. Gen. Microbiol. 73, 13-22.

de Vries, O.M.H., and Wessels, J.G.H. 1973. Release of protoplasts from Schizophyllum commune by combined action of purified a-1,3-glucanase and chitinase derived from Trichoderma viride. J. Gen. Microbiol. 76, 319-330.

de Vries, O.M.H., and Wessels, J.G.H. 1975. Chemical analysis of cell wall regeneration and reversion of protoplasts from Schizophyllum commune. Arch. Microbiol. 102, 209-218.

Delcaire, J.R. 1978. Economics of edible mushrooms. In: The Biology and Cultivation of Edible Mushrooms. pp. 727-793. Edited by Chang, S.T., and Hayes, W.A. Academic Press, New York.

Desborough, S., and Peloquin, S.T. 1968. Potato variety identification by use of electrophoretic patterns of tubers proteins of enzymes. J. Exp. Bot. 18, 100-109.

Deutch, C.E., and Parry, J.M. 1974. Sphaeroplast formation in yeast during the transition from exponential phase to stationary phase. J. Gen. Microbiol. 80, 259-268.

Dooijewaard-Kloosterziel, A.M.P., Sietsma, J.H., and Wouters, J.T. M. 1973. Formation and regeneration of Geotrichum protoplasts. J. Gen. Microbiol. 74, 205-209.

Dudits, D., Hadlaczky, G ,Bajszar, G.Y., Koncz, C.S., Lazar, D.,

and Horvath, G. 1979. Plant regeneration from intergeneric cell hybrids. Plant Sc., Lett. 15, 101-112.

Duell, E.A., Inoue, S., and Utter, M.F. 1964. Isolation and properties of intact mitochondria from spheroplasts of yeast. J. Bacteriol. 88, 1762-1773.

Eddy, A.A., and Williamson, D.H. 1957. A method of isolating protoplasts from yeast. Nature (London) 179, 1251-1253.

Eddy, A.A., and Williamson, D.H. 1959. Formation of aberrant cell walls and of spores by the growing yeast protoplast. Nature (London) 183, 1101-1102.

Eger, G. 1978. Biology and breeding of Pleurotus. In: The Biology and Cultivation of Edible Mushrooms. pp. 497-520. Edited by Chang, S.T., and Hayes, W.A. Academic Press, New York.

Eger, G., Eden, G., and Wissig, E. 1976. Pleurotus ostreatus-Breeding potential of a new cultivated mushroom. Theor. Appl. Genet. 47, 155-163.

Eger, G., Li, S.F., and Hermilo, L.-L. 1979. Contribution to the discussion on the species concept in the Pleurotus ostreatus complex. Mycologia 71, 577-588.

Ellingboe, A.H. 1964. Nuclear migration in dikaryotic-homokaryotic matings in Schizophyllum commune. Amer. J. Bot. 51, 133-139.

Ellingboe, A.H., and Raper, J.R. 1962. The Buller Phenomenon in Schizophyllum commune : nuclear selection in fully compatible dikaryotic-homokaryotic matings. Amer. J. Bot.

49, 454-459.

Elliott, T.J. 1978. Comparative Sexuality in Agaricus species. J. Gen. Microbiol. 107, 113-122.

Elliott, T.J. 1982. Genetics and breeding of cultivated mushrooms. In: Tropical Mushrooms - Biological Nature and Cultivation Methods. pp. 11-30. Edited by Chang, S.T., and Quimio T.H. The Chinese University Press, Hong Kong.

Emerson, S., and Emerson, M.R. 1958. Production, reproduction and reversion of protoplast like structures in the osmotic strain of Neurospora crassa. Proc. Natl. Acad. Sci. USA 44, 668-671.

Esser. K., and Blaich, R. 1973. Heterogenic incompatibility in plants and animals. Adv. Genert. 17,107-152.

Esser, K., and Kuenen, R. 1967. Genetics of Fungi. Berlin-Heidelbery - New York, Springer.

Esser, K., and Meinhardt, F. 1977. A common genetic control of dikaryotic and monokaryotic fruiting in the basidiomycete Agrocybe aegerita. Mol. Gen. Genet. 155, 113-115.

Eugenio, C.P., and Anderson, N.A. 1968. The genetics and cultivation of Pleurotus ostreatus. Mycologia 60, 627-634.

Fawcett, P.A., Loder, P.B., Duncan, M.J., Baesley, T.S., and Abraham, E.P. 1973. Formation and properties of protoplasts from antibiotic-producing strains of Penicillium chrysogenum and Cephalosporium acremorium. J. Gen. Microbiol. 79, 293-309.

Ferenczy, L., Kevei, F., and Szegedi, M. 1974. Increased fusion

frequency of Aspergillus nidulans protoplasts. Experimentia 31, 50-52.

Ferenczy, L., Kevei, F., and Szegedi, M. 1975. High-frequency fusion of fungal protoplasts. Experimentia 31, 1028-1030.

Ferenczy, L., Kevei, F., and Szegedi, M. 1976. Fusion of fungal protoplasts induced by polyethylene glycol. In: Microbial and Plant Protoplasts. pp. 177-188. Edited by Peberdy, J.F., Rose, A.H., Rogers, H.J., and Cocking, E.C. Academic Press, London.

Ferenczy, L., Kevei, F., and Zsolt, J. 1974. Fusion of protoplasts. Nature (London) 248, 793-794.

Ferenczy, L., and Maraz, A. 1977. Transfer of mitochondria by protoplast fusion in Saccharomyces cerevisiae. Nature (London) 268, 524-525.

Ferenczy, L., Szegedi, M., and Kevei, F. 1977. Interspecific protoplast fusion and complementation in Aspergilli. Experimentia 33, 184-186.

Fincham, J.R.S., and Day, P.R. 1965. Fungal Genetics. Davis Co., Philadelphia.

Fodor, K., and Alzoldi, L. 1976. Fusion of protoplasts of Bacillus megaterium. Proc. Nat. Acad. Sci., U.S.A. 73, 2147-2150.

Fournier, P., Provost, A., Bourgnignon, C., and Heslot, H. 1977. Recombination after protoplast fusion in the yeast Candida tropicalis. Arch. Microbiol. 115, 143-149.

Foury, F., and Groffeau, A. 1973. Combination of 2-deoxyglucose

and snail-gut treatments for preparing sphaeroplasts of Schizosaccharomyces pombe. J. Gen. Microbiol. 75, 227-229.

Gander, J.E. 1974. Fungal cell wall glycoproteins and peptidopolysaccharides. Ann. Rev. Microbiol. 28, 103-119.

Garcia-Acha, I., Lopez-Belmonte, F., Villanueva, J.R. 1966. Regeneration of mycelial protoplasts of Fusarium culmorum. J. Gen. Microbiol. 45, 515-523.

Gascon, S., and Villanueva, J.R. 1965. Magnesium sulphate as stabilizer during liberation of yeast and mould protoplasts. Nature (London) 205, 822-823.

Gleba, Y.Y., and Hoffmann, F. 1978. Hybrid cell lines Arabidopsis thaliana + Brassica campestris : no evidence for specific chromosome elimination. Mol. Gen. Genet. 165, 257-264.

Gleba, Y.Y., and Hoffmann, F. 1980. "Arabidobrassica" : a novel plant obtained by protoplast fusion. Planta 149, 112-117.

Gleba, Y.Y., Momot, Y.P., Cherep, N.N., and Skarzynskaya, M.V. 1982. Intertribal hybrid cell lines of Atropa belladonna x Nicotiana chinensis obtained by cloning individual protoplast fusion products. Theor. Appl. Genet. 62, 75-79.

Gorini, L., and Kaufman, H. 1960. Selecting bacterial mutants by the penicillin method. Science 131, 604.

Grimsley, N.H., Ashton, N.W., and Cove, D.J. 1977. The production of somatic hybrids by protoplast fusion in moss, Physcomitrella patens. Mol. Gen. Genet. 154, 97-100.

Grout, B.W.W., and Coutts, R.H.A. 1974. Additives for the

enhancement of fusion and endocyctosis in higher plant protoplasts: an electrophoretic study. Plant Sci. Lett. 2, 397-403.

Hamlyn, P.F., Bradshaw, R.E., Mellon, F.M., Santiago, C.M., Wilson, J.M., and Peberdy, J.F. 1981. Efficient protoplast isolation from fungi using commercial enzymes. Enzyme Microb. Technol. 3, 321-325.

Han, Y.H., Chen, K.M., and Cheng, S. 1974. Characteristics and cultivation of new Pleurotus in Taiwan. Mush. Sci. 9, 167-173.

Harms, C. T., and Potrykus, I. 1978. Fractionation of plant protoplast types by iso-osmotic density gradient centrifugation. Theor. Appl. Genet. 53, 57-63.

Hartmann, J.X., Kao, K.N., Gamborg, O.L., and Miller, R.A. 1973. Immunological methods for the agglutination of protoplasts from cell suspension cultures of different genera. Planta 112, 45-56.

Hein, T., Przewezny, T., and Schieder, O. 1983. Culture and selection of somatic hybrids using an auxotrophic cell line. Theor. Appl. Genet. 64, 119-122.

Hoess, R.H., Smith, H.H., and Stowell, C.P. 1974. A genetic analysis of peroxidase isozymes in two species of Nicotiana. Biochem. Genet. 11, 319-323.

Holliday, R. 1956. A new method for the identification of biochemical mutants of micro-organisms. Nature (London) 178, 987.

Hou, H.H., and Wu, L.C. 1972. Nuclear behavior of cultivated mushroom. Bot. Bull. Acad. Sinic. (Taiwan) 13,82-91.

Houssett, P., Nagy, M., and Schwencke, J. 1975. Protoplasts of Schizosaccharomyces pombe: an improved method for their preparation and the study of their guanine uptake. J. Gen. Microbiol. 90, 260-264.

Hunter, R.L., and Markert, C.L. 1957. Histochemical demonstration of enzymes separated by zone electrophoresis in starch gels. Science 125, 1294.

Iglesias, L., Lima, H., and Simon, J.P. 1974. Isoenzyme identification of zygotic and nucellar seedlings in Citrus. J. Hered. 65, 81-84.

Jandaik, C.L., and Kapoor, J.N. 1976. Studies on cultivation of Pleurotus sajor-caju (Fr.) Singer. Mush. Sci. 9, 667-672.

Kameya, T. 1973. The effects of gelatin on aggregation of protoplasts from higher plants. Planta 115, 77-82.

Kao, K.N. 1977. Chromosomal behaviour in somatic hybrids of soybean-Nicotiana glauca. Mol. Gen. Genet. 150, 225-230.

Kao, K.N., and Michayluk, M.R. 1974. A method for high-frequency intergeneric fusion of plant protoplasts. Planta 115, 355-367.

Kemp, R.F.O. 1975. Breeding biology of Coprinus species in the section Lanatuli. Trans Br. Mycol. Soci. 65, 375-388.

Kevei, F., and Peberdy, J.F. 1977. Interspecific hybridization between Aspergillus nidulans and Aspergillus rugulosus by

fusion of somatic protoplasts. J. Gen. Microbiol. 102, 255-262.

Kevei, F., and Peberdy, J.F. 1979. Induced segregation in interspecific hybrids of Aspergillus nidulans and Aspergillus rugulosus obtained by protoplast fusion. Mol. Gen. Genet. 170, 213-218.

Kimura, K. 1958. Diploidization in the Hymenomycetes. II. Nuclear behavior in the Buller Phenomenon. Biol. J. Okayama Univ. 4, 1-59.

Kitani, Y., and Olive, L.S. 1967. Genetics of Sordaria fimicola. VI. Gene conversion at the g locus in mutant x wild type crosses. Genetics 57, 767-782.

Kitani, Y., and Olive, L.S. 1969. Genetics of Sordaria fimicola. VII. Gene conversion at the G locus in interallelic crosses. Genetics 62, 23-66.

Krumbiegel, G., and Schieder, O. 1979. Selection of somatic hybrids after fusion of protoplasts from Datura innoxia and Atropa belladonna. Planta 145, 371-475.

Kurtzmun, R.H. Jr., and Zadrazil, F. 1982. Physiological and taxonomic considerations for cultivation of Pleurotus mushrooms. In: Tropical Mushrooms: Biological Nature and Cultivation Methods. pp. 299-348. Edited by Chang, S.T., and Quimio, T.H. The Chinese University Press, Hong Kong.

Kut, S.A., and Evans, D.A. 1984. ADH isozymes in seed of Nicotiana species and somatic hybrids. J. Hered. 75, 215-

Li, S.F. 1980. Studies on the Tolerance to Elevated Temperatures in Pleurotus ostreatus (Jacq. ex Fr.) Kummer. Bibliotheca Mycologica, Band 76. Cramer. Vaduz.

Lo Schiavo, F., Mela, L., Nuti Ronchi, V., and Terzi, M. 1980. Electrophoretic mobility of isozymes from different plant species and its possible use in identifying cell hybrids. Plant Sci. Lett. 18, 45-55.

Lonnendonker, N., and Schieder, O. 1980 Amylase isoenzymes of the genus Datura as a simple method for an early identification of somatic hybrids. Plant Sci. Lett. 17, 135-139.

Lopez-Belmonte, F., Garcia Acha, I., Villanueva, J. R. 1966. Observation on the protoplasts of Fusarium culmorum and on their fusion. J. Gen. Microbiol. 45, 127-134.

MacDonald, K.D. 1968. The selection of auxotrophs of Penicillium chrysogenum with nystatin. Genet. Res. 11, 327-330.

Mahmoud, S.H., Gatehouse, J.A., and Boulter, D. 1984. Inheritance and mapping of isoenzymes in pea (Pisum sativum L.). Theor. Appl. Genet. 68, 559-566.

Markert, C.L., 1968. The molecular basis for isozymes. Ann. N.Y. Acad. Sci. 151, 14-40.

Markert, C.L., and Moller, F. 1959. Multiple forms of enzymes : Tissue, ontogenetic, and species specific patterns. Proc. Natl. Acad. Sci. USA 45, 753 - 763

Marquis, R.E., and Corner, T.R. 1976. Isolation and

properties of bacterial protoplasts. In: Microbial and Plant Protoplasts. pp. 1-22. Edited by Perberdy, J.F., Rose, A.H., Rogers, H.J., and Cocking, E.C. Academic Press, New York.

Martin, H.H. 1983. Protoplasts and spheroplasts of Gram-negative bacteria with special emphasis on Proteus mirabilis. In: Protoplasts, 1983 Lecture Proceedubgs 6t International Protoplast Symposium. pp. 213-226. Edited by Potrykus, I., Harms, C.T., Hinnen, A., Hutter, R., King, P.J., and Shillito, R.D. Birkhause, Basel.

May, B., and Royse, D.J. 1981. Application of the electrophoretic methodology to the elucidation of genetic life histories of edible mushrooms. Mush. Sci. 11, 799-817.

May, B., and Royse, D.J. 1982. Confirmation of crosses between lines of Agaricus brunnescens by isozyme analysis. Exp. Mycol. 6, 283-292.

Meinhardt, F., and Esser, K. 1981. Genetic studies of the Basidiomycete Agrocybe aegerita; genetic control of fruit body formation and its practical implications. Theor. Appl. Genet. 60, 265-268.

Meyer, K.H. 1952. Natural and synthetic high polymers. Interscience, New York.

Meyer, Y., and Abel, N.O. 1975. Importance of the wall for cell division and in the activity of the cytoplasma in cultured tobacco protoplasts. Planta 123, 33-40.

Moore, D. 1969. The mutagenic action of N-methyl-N-nitro-N-nitrosoguanidine on Coprinus lagopus. J. Gen. Microbiol. 55, 121-125.

Moore, D. 1975. Production of Coprinus protoplasts by use of chitinase or helicase. Trans. Br. Mycol. Soc. 65, 134-136.

Moore, P.M., and Peberdy, J.F. 1976. Release and regeneration of protoplasts from the conidia of Aspergillus flavus. Trans. Br. Mycol. Soc. 66, 421-425.

Morgan, A.J. 1983. Yeast strain improvement by protoplast fusion and transformation. In: Protoplasts, 1983 Lecture Proceedings 6th International Protoplast Symposium. pp. 155-166. Edited by Protrykus, I., Harms, C.T., Hinnen, A., King, P.J., and Shilito, R.D. Birkhauser Verlag, Boston.

Morris, N.R. 1978. Preparation of large molecular weight DNA from the fungus Aspergillus nidulans. J. Gen. Microbiol. 106, 387-389.

Nagata, T., and Takebe, I. 1970. Cell wall regeneration and cell division in isolated tobacco mesophyll protoplasts. Planta 92, 301-308.

Ohnuki, T., Etoh, Y., and Heppu, T. 1982. Intraspecific and interspecific hybridization of Mucor pusillus and M. miehei by protoplast fusion. Agric. Biol. Chem. 46, 451-458.

Olive, L.S. 1956. Genetics of Sordaria fimicola. I. Ascospore color mutants. Amer. J. Bot. 43, 97-107.

Papazian, H.P. 1949. The incompatibility factors in Schizophyllum commune (abs.). Amer. J. Bot. 39, 813.

Papazian, H.P. 1950. Physiology of the incompatibility factors in Schizophyllum commune. Bot. Gaz. 112, 143-163.

Papazian, H.P. 1951. The incompatibility factors and a related gene in Schizophyllum commune. Genetics 36, 441-459.

Patnaik, G., Cocking, E.C., Hamill, J., and Pental, D. 1982. A simple procedure for the manual isolation and identification of plant heterokaryons. Plant Sci. Lett. 24, 105-110.

Peberdy, J.F. 1976. Isolation and properties of protoplasts from filamentous fungi. In: Microbial and Plant Protoplasts. pp. 39-50. Edited by Peberdy J.F., Rose, A.H., Roger, H.J., and Cocking, E.C. Academic Press, London.

Peberdy, J.F. 1979. Fungal protoplasts; isolation, reversion and fusion. Ann. Rev. Microbiol. 33, 21-39.

Peberdy, J.F. 1980. Protoplast fusion - a tool for genetic manipulation and breeding in industrial microorganisms. Enzyme Microb. Technol. 2, 23-29.

Peberdy, J.F., Buckley, C.H., Daltrey, D.C., and Moore, P.M. 1976. Factors affecting protoplast release in some filamentous fungi. Trans. Br. Mycol. Soc. 67, 23-26.

Peberdy, J.F., Buckley, C.E., and Gilson, R.K. 1973. Studies on the regeneration of Aspergillus nidulans. In: Yeast, Mould and Plant Protoplast. pp. 285-294. Edited by Villanueva, J.R., Garcia-Acha, I., Gascon, S., and Urubaru, F. Academic

Press, London.

Peberdy, J.F., Essyen, H., and Anne, J. 1977. Interspecific hybridization between Penicillium chrysogenum and Penicillium cyaneo-fulvum following protoplast fusion. Mol. Gen. Genet. 157, 281-284.

Peberdy, J.F., Gibson, R.K., 1971. Regeneration of Aspergillus nidulans protoplasts. J. Gen. Microbiol, 69, 325-330.

Peberdy, J.F., and Isaac, S., 1976. An improved procedure for protoplast isolation from Aspergillus nidulans. Microbios Lett. 3, 7-9.

Perkins, D.D. 1949. Biochemical mutants in the smut fungus Ustilago maydis. Genetics 34, 607-626.

Potrykus, I., and Hoffmann, F. 1973. Transplantation of nuclei into protoplasts of higher plants. Z. Pflanzenphysiol. 69, 287-289.

Power, J.B., Cummins, S.E., and Cocking, E.C. 1970. Fusion of isolated plant protoplasts. Nature (London) 225, 1016-1018.

Power, J.B., Frearson, E.M., Hayward, C., George, D., Evans, P.K., Berry, S.F., and Cocking, E.C. 1976. Somatic hybridization of Petunia hybrida and P. parodii. Nature (London) 263, 500-502.

Provost, A., Bourguignon, C., Fournier, P., Ribet, A.M., and Heslot, H. 1978. Intergeneric hybridization in yeasts throuh protoplast fusion. FEMS Microbiol. Lett. 3, 309-

312.

Qiu, J.Y., Liao, H.C., and Wu, Y.C. 1982. Preparation of an enzyme for lysing the cell wall of higher basidiomycete. Hereditas (Beijing). 4, 13-14. (In Chinese).

Raper, C. A. 1978. Control of development by the incompatibility system in Basidiomycetes. In: Genetics and Morphogenesis in the Basidiomycetes. pp. 3-30. Edited by Schwald, M.N., and Miles, P.G. Academic Press, New York.

Raper, C.A., and Raper, J.R. 1966. Mutations modifying sexual morphogenesis in Schizophyllum. Genetics 54, 1151-1168.

Raper, C.A., and Raper, J.R. 1973. Mutational analysis of a regulatory gene for morphogenesis in Schizophyllum. Proc. Natl. Acad. Sci. USA 70, 1427-1431.

Raper, C.A., Raper, J.R., and Miller, R.E. 1972. Genetic analysis of the life cycle of Agaricus bisporus. Mycologia 64, 1088-1117.

Raper, J.R. 1966. Genetics of sexuality in Higher Fungi. Ronald Press, New York.

Raper, J.R., Boyd, D.H., and Raper, C.A. 1965. Primary and secondary mutations at the incompatibility loci in Schizophyllum. Proc. Natl. Acad. Sci. USA 53, 1324-1332.

Raper, J.R., and Miles, P.C. 1958. The genetics of Schizophyllum commune. Genetics 43, 530-546.

Raudaskosky, M., Stamberg, J., Baunik, N., and Koltin, Y. 1976. Mutational analysis of natural alleles at the B incompatibility factor of Schizophyllum commune: a2 and b6.

Genetics 83, 507-510.

Rodriguez Aguirre, M.J., and Villanueva. 1962. Production of protoplast-like structures from various species of fungi. Nature (London) 196, 693-694.

Rowlands, T.R., and Turner, G. 1973. Nuclear and extranuclear inheritance of oligomycin resistance in Aspergillus nidulans. Mol. Gen. Genet. 126, 201-206.

Roxon, J.E., and Jong, S.C. 1977. Sexuality of an edible mushroom, Pleurotus sajor-caju. Mycologia 69, 203-205.

Royse, D.J., and May, B. 1982a. Genetic relatedness and its application in selective breeding of Agaricus brunnescens. Mycologia 74, 569-575.

Royse, D.J., and May, B. 1982b. Use of isozyme variation to identify genotypic classes of Agaricus brunnescens. Mycologia 74, 93-102.

Ruiz-Herrera, J., and Bartnicki-Garcia, S. 1976. Proteolytic activation and inactivation of chitin synthetase from Mucor rouxii. J. Gen. Microbiol. 97, 241-249.

Santiago, C.M., Jr. 1982a. Studies on the physiology and genetics Volvariella volvarea (Bull. ex Fr.) Sing. Ph.D. Thesis, University of Nottingham, England. 238pp.

Santiago, C.M., Jr. 1982b. Production of Volvariella protoplasts by use of Trichoderma enzyme. Mush. Newslett. Trop. 3, 3-6.

Scandalios, J.C. 1969. Genetic control of multiple molocular forms of enzymes in plants: A review. Biochem. Genet. 3,

37-79.

Scandalios, J.G. 1968. Alcohol dehydrogenase in maize: Genetic basis for isozymes. Science 166, 623-624.

Scandalios, J.G. 1974. Isozymes in development and differentiation. Ann. Rev. Plant Physiol. 25, 225-258.

Schaeffer, P., Cami, B., Hotchkiss, R.D. 1976. Fusion of bacterial protoplasts. Proc. Natl. Acad. Sci. USA 73, 2151-2155.

Schenk, R.U., and Hildebrandt, A.C. 1968. Somatic hybridization: A new approach to genetic change. Amer. J. Bot. 55, 731.

Schieder, O. 1975. Selection of a somatic hybrid between auxotrophic mutants of Sphaerocarpos donnellii Aust. using the method of protoplast fusion. Z. Pflanzenphysiol. 74, 357-365.

Schwartz, D. 1964. Genetic studies on mutant enzymes in maize, V. In vitro interconversion of allelic isozymes. Proc. Natl. Acad. Sci. USA 52, 222-226.

Schwartz, D. 1966. The genetic control of alcohol dehydrogenase in maize: gene duplication and repression. Proc. Nalt. Acad. Sci. USA 56, 1431-1436.

Schwartz, D., and Endo, T. 1966. Alcohol dehydrogenase polymorphism in maize—simple and compound loci. Genetics 53, 709-715.

Schwartz. D., Fuchsman, L., and McGrath, K.H. 1965. Allelic isozymes of the pH 7.5 esterase in maize. Genetics 52,

1265-1268.

Santandreu, R., Herrero, E., Elorza, M.V ., Rico, H., and Pastor, J. 1983. Synthesis and assembly of wall polymers on regenerating yeast protoplasts. In: Protoplasts 1983 Lecture Proceedings 6th International Protoplast Symposium. pp 187-196. Edited by Potrykus, I., Harms, C.T., Hinnen, A., King, P.J., and Shillito, R.D. Birkhauser Verlag, Boston.

Setlow, J.K., Boling, M.E., and Bollum, F.J. 1965. The chemical nature of photoreactivable lesions in DNA. Proc. Natl. Acad. Sci. USA 53, 1430-1436.

Setlow, R.B., Carrier, W.L. 1964. The disappearance of thymine dimers from DNA error-correcting mechanism. Proc. Natl. Acad. Sci. USA 51, 226-231.

Setlow, R.B., Carrier, W.L., Bollum, F.J. 1965. Pyrimidine dimers in UV-irradiated polydI:dC. Proc. Natl. Acad. Sci. USA 53, 1111-1118.

Setlow, J.K., and Setlow, R.B. 1963. Nature of the photoreactivable ultraviolet lesion in deoxyribonucleic acid. Nature (London) 197, 560-562.

Shahin, M.M. 1972. Relationship between yield of protoplast and growth phase in Saccharomyces. J. Bacteriol. 110, 769-771.

Shannon, L.M. 1968. Plant isoenzymes. Ann. Rev. Plant Physiol 19, 187-210.

Shepard, J.F., Bidney, D., Barsby, T., and Kemble, R. 1983. Genetic transfer in plants through interspecific protoplast

fusion. Science 219, 683-688.

Shneyour, A., and Avron, M. 1975. A method for producing, selecting and isolating photosynthetic mutants of Euglena gracilis. Plant Physiol. 55, 142-144.

Shneyour, Y., Stamberg, J., Hundert, P., Werczberger, R., and Koltin, Y. 1978. Selection with cycloheximide of metabolic and UV-sensitive mutants of Schizophyllum commune and Saccharomyces cerevisiae. Mut. Res. 49, 195-201.

Sidorov, V.A., and Maliga, P. 1982. Fusion-complementation analysis of auxotrophic and chlorophyll-deficient lines isolated in haploid Nicotiana plumbaginifolia protoplast culture. Mol. Gen. Genet. 186, 328-332.

Sietsma, J.H., and de Boer, W.R. 1973. Formation and regeneration of protoplasts from Pythium PRL2142. J. Gen. Microbiol. 74, 211-217.

Singer, R. 1975. The Agaricales in Modern Taxonomy. 3rd ed. Vaduz: J. Cramer.

Sipiczki, M. 1979. Interspecific protoplast fusion in fission yeasts. Curr. Microbiol. 3, 37-40.

Sipiczki, M., and Feranczy, L. 1977a. Fusion of Rhodosporidium protoplasts. FEMS Microbiol. Lett. 2, 203-205.

Sipiczki, M., and Feranczy, L. 1977b. Protoplast fusion of Schizosaccharomyces pombe auxotrophic mutants of identical mating-type. Mol. gen. Genet. 151, 77-81.

Sommer, A., and Lewis, M.J. 1971. Effect of dithiothreitol on yeast: Spheroplast formation and invertase release. J. Gen.

Microbiol. 68, 327 - 335.

Spencer, J.F.T., Laud, P., and Spencer, D.M. 1980. The use of mitochondrial mutants in the isolation of hybrids involving industrial yeast strains. II. Use in isolation of hybrids obtained by protoplast fusion. Mol. Gen. Genet. 178, 651-654.

Spencer, J.F.T., and Spencer, D.M. 1981. The use of mitochondrial mutants in hydridization of industrial yeasts. III. Restoration of mitochondrial funktion in petites of industrial yeast strains by fusion with respiratory-competent protoplasts of other yeast species. Curr. Genet. 4, 177-180.

Spencer, J.F.T., and Spencer, D.M. 1983. Genetic improvement of industrial yeasts. Ann. Rev. Microbiol. 37, 121-142.

Stahl, U. 1978. Zygote formation and recombination between like mating types in the yeast Saccharomycopsis lipolytica by protoplast fusion. Mol. Gen. Genet. 160, 111-113.

Stahl, U., and Esser, K. 1976. Genetics of fruit body production in higher basidiomycetes. I. Monokaryotic fruiting and its correlation with dikaryotic fruiting in Polyporus ciliatus. Mol. Gen. Genet. 148, 183-197.

Stanley, V.C., and English, M.P. 1965. Some effects of nystatin on the growth of four Aspergillus species. J. Gen. Microbiol. 40, 107-118.

Stewart, G.G. 1981. The genetic manipulation of industrial yeast strains. Can. J. Microbiol. 27, 973-990.

Svoboda, A. 1978. Fusion of yeast protoplasts induced by polyethylene glycol. J. Gen. Microbiol. 109, 169-175.

Swiezynski, K.M., and Day, P.R. 1960. Heterokayron formation in Coprinus lagopus. Genet. Res. Camb. 1, 114-128.

Takebe, I. 1975. The use of protoplasts in plant virology. Ann. Rev. Phytopathol. 13, 105-125.

Takemaru, T., and Kamada, T. 1971. Gene control of basidiocarp development in Coprinus macrorhizus. Rep. Tottori Myc. Inst. (Japan) 9, 21-35.

Tatum, E.L., Barratt, R.W., Fries, N., and Bonner, D. 1950. Biochemical mutant strains of Neurospora produced by physical and chemical treatment. Amer. J. Bot. 37, 38-46.

Terakawa, H. 1957. The nuclear behavior and the morphogenesis in Pleurotus ostreatus. Sci. Pap. Coll. Gen. Educ., Univ. Tokyo 7, 61-88.

Toby, A.L., and Kemp, C.L. 1975. Mutant enrichment in the colonial alga, Eudorina elegans. Genetics 81, 243-251.

Torres, A.H., Diedenhofen, U., Bergh, B.O., and Knight, R.J. 1978. Enzyme polymorphism as genetic markers in the avocado. Amer. J. Bot. 65, 134-139.

Van Solingen, P., and Van der Plaat, J.B. 1977. Fusion of yeast protoplasts. J. Bacteriol. 130, 946-947.

Vandendries, R. 1933. De la valeur du barrage sexuel, comme criterium dans l'analyse d'une sporee tetrapolaire de basidiomycete: Pleurotus ostreatus. Genetica 15, 202-212.

Villanueva, J.R., and Garcia-Acha, I. 1971. Production and use of

fungal protoplasts. In: Methods in Microbiology. Vol. 4, pp. 665-718. Edited by Booth, C. Academic Press, New York.

Vogel, H.J. 1964. Distribution of lysine pathways among fungi: Evolutionary implications. Amer. Nat. 98, 435-446.

Wacker, A., Dellweg, H., and Jacherts, D. 1962. Thymine dimerization and survival of bacteria. J. Mol. Biol. 4, 410-412.

Wagner, G.J., and Siegelman, H.W. 1975. Large-scale isolation of intact vacuoles and isolation of chloroplasts from protoplasts of mature plant tissues. Science 190, 1298-1299.

Wallin, A., Glimelius, K., and Eriksson, T. 1974. The induction of aggregation and fusion of Daucus carota protoplasts by polyethylene glycol. Z. pflanzenphsiol. 74, 64-80.

Wang, S.S., and Anderson, N.A. 1972. A genetic analysis of sporocarp production in Pleurotus sapidus. Mycologia 64, 521-528.

Watson, J.D. 1975. Molecular Biology of the Gene. W.A. Benjamin, London.

Weingberg, R., and Orton, W.L. 1965. Elution of acid phosphatase from the cell surface of Saccharomyces mellis by potassium chloride. J. Bacteriol. 90, 82-90.

Wetter, L.R. 1977. Isoenzyme patterns in soybean - Nicotiana

somatic hybrid cell lines. Mol. Gen. Genet. 150, 231-235.

Wetter, L.R. 1982. Isozyme analyses of cultured plant cells. In: Plant Tissue Culture Methods. 2nd ed. pp. 105-111. Edited by Wetter, L.R., and Constabel, F. National Research Council of Canada, Saskatchewan, Canada.

Whittaker, P.A., and Leach, S.M. 1978. Interspecific hybrid production between the yeasts Kluyveromyces lactis and Kluyveromyces fragilis by protopolast fusion. FEMS Microbiol. Lett. 4, 31-34.

Withers, L., and Cocking, E.C. 1972. Fine structural studies on spontaneous and induced fusion of higher plant protoplasts. J. Cell Sci. 11, 59-75.

Witkin, E.M. 1969. Ultraviolet-induced mutation and DNA repair. Ann. Rev. Genet. 3, 525-553.

Wood, R.A. 1973. Biochemical Genetics. Chapman and Hall, London.

Wright, W.E. 1978. The isolation of heterokaryons and hybrids by a selective system using irreversible biochemical inhibitor. Exp. Cell Res. 112, 395-407.

Young, J.D., Gorman, J.W., Gorman, J.A., and Bock, R.M. 1976. Indirect selection for auxotrophic mutants of Saccharomyces cerevisiae using the antibiotic netropsin. Mut. Res. 35, 423-428.

Yusof. N., and Graham, K.M. 1977. A preliminary genetic study of a local edible mushroom, Pleurotus flabellatus (Berk. and BR.) Sacc. Mol. Appl. Biol. 6, 59-66.

Zadrazil, F. 1976. The ecology and industrial production of Pleurotus ostreatus, Pleurotus Florida, Pleurotus cornucopial and Pleurotus eryngii. Mush. Sci. 9, 621-652.

Zadrazil, F., and Kurtzman, R.H. Jr. 1982. The biology of Pleurotus cultivation in the tropics. In: Tropical Mushrooms - Biological Nature and Cultivation Methods. pp. 277-298. Edited by Chang, S.T., and Quimio, T.H. The Chinese University Press, Hong Kong.

Zimmermmann, U., and Scheurich, P. 1981. High frequency fusion of plant protoplasts by electric fields. Planta 151, 26-32.